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Analysis of *FRUITFULL* promoter motifs and their influence on valve expression during fruit development

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The Thesis of Jennifer C. Woods is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

Analysis of *FRUITFULL* promoter motifs and their
influence on valve expression during fruit development

by

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FRUITFULL (*FUL*) is a member of the MADS box family of genes that is involved with promoting the growth of carpe valves after fertilization. In *Arabidopsis thaliana*, *FUL* plays a fundamental role in fruit development, as *ful* fruit fail to elongate and are indehiscent. *FUL* is typically expressed throughout the floral meristem and vasculature early on, with a notable "apical-basal expression gradient" that develops along valve tissue after fertilization. The particular distribution of *FUL* activity in valves is likely correlated to its function in valve development, with deviant expression patterns causing severe defects in fruit morphology. Though previous studies have identified a 3.9 kb promoter region capable of driving wild type-like *FUL* expression, little is known about individual regulatory motifs that are responsible for patterning this expression.

A series of promoter-*GUS* fusions were generated that allows the comparison of *FUL* transcriptional activity when driven by different promoter elements. The

SQUAMOSA promoter-Binding Protein (SBP) motif, located 2971 bp upstream the *FUL* start codon, was found to be crucial for *FUL*'s valve-specific expression, though not sufficient in maintaining the WT-like bipolar expression gradient. Two perfect CArG-box motifs (located 2038 bp and 2512 bp upstream *FUL* ATG) redundantly regulate *FUL* expression, with the presence of at least one functional CArG-box necessary to initiate *FUL* transcription. Additionally, two auxin response elements (AuxREs) were identified at either end of the 3.9 kb promoter (641 bp and 3679 bp upstream *FUL* ATG) and function as negative regulators of *FUL* transcription.

INTRODUCTION

"An apple a day keeps the doctor away," a popular children's rhyme, is often told to encourage youngsters to eat their fruits and vegetables. From apples and oranges to tomatoes and eggplant, the cornucopia of vitamins and minerals packed into fruit make them a valuable part of our diet, especially for young, developing bodies. Fruits can be consumed in their natural state (such as bananas and peaches) or processed into alternate forms (such as juices, alcohols, oils, and preserves). Their high content of vitamins, minerals, phenols, and fiber have been shown to prevent disorders such as cardiovascular disease, Alzheimer's disease, cataracts and diabetes, as well as reducing one's risk of cancer. It comes as no surprise that fruit and plant extracts are commonly used in pharmaceutical practices and holistic medicines, such as the use of morphine (derived from opium poppy) for pain relief.

While the nutritional benefits of fruit are widely acknowledged, there are many other everyday applications that are taken for granted. The physical characteristics of fruit, such as the texture or color of its tissue, can be utilized for the production of utensils (e.g. water jugs made from dried and hollowed gourds), natural dyes (e.g. cherry, mulberry), candles (e.g. wax from bayberries), clothing (e.g. hemp or coconut fiber), and cosmetics (e.g. perfumes and skin products).

Fruits can even play spiritual or cultural roles in some societies, such as the American tradition of carving pumpkins for Halloween. As you can see, the applications of fruit are endless, though our knowledge of their life cycle is limited. The immense

pragmatism of fruit studies, along with its potential to improve the quality of life, has made fruit development research an increasingly popular subject as of late. Understanding how different fruits develop these "utilizable qualities" will help uncover more extensive and efficient means of exploiting such qualities, and have a positive impact on modern agriculture and economy.

Thousands of different species of plants that exist today, yielding a very diverse set of practical functions, yet the formation of fruit serves the same purpose for all – all evolved specific structures to optimize seed dispersal and maximize reproductive success (Robles and Pelaz, 2005; Roeder and Yanofsky, 2006). For instance, fruits such as the cocklebur depend on surrounding animals for their seed dispersal and, thus, developed barbed-like structures around their fruit that attach to the coats of animals and are carried to new locations. Other fruits, such as berries, have appealing colors or sweet flesh that attract animals to consume them and deposit the seeds in a natural fertilizer later (Roeder and Yanofsky, 2006). Many dry fruits rely on the force of wind for seed dispersal. Such fruits evolved long, thin structures like helicopter blades (e.g. as in maple) or tiny parachutes (e.g. in dandelions) that can maximize dispersal distance by wind.

Since the fruit plays such a critical role in a plant's life cycle and reproductive success (Robles and Pelaz, 2005; Roeder and Yanofsky, 2006), it is not surprising that it is the most complex structure in plant anatomy, consisting of many distinct cell types (Robles and Pelaz, 2005; Ferrandiz et al., 1999). The study of these specialized tissues and how they are formed has been a growing field in biology within the last decade, with many milestones made by Prof. Martin F. Yanofsky's lab at the University of California,

San Diego. Though much is still unknown about the factors that control fruit development, we are taking gradual steps in the right direction to fill in the missing information.

Arabidopsis thaliana, a Model Organism

Classical genetic studies tend to focus on a single model species for experimentation, whose properties are conducive to the area of interest and whose subsequent findings may be applied to the majority of related species in that family. For example, tomatoes are often used in experiments for plant development research, with an emphasis on fleshy fruits. *Arabidopsis thaliana*, a small mustard weed in the Brassicaceae family, is widely used for studies on dehiscent fruit. Dehiscent fruit are those that break open to release seeds (while a part of it stays intact with the mother plant), as opposed to indehiscent fruit whose seeds remain enclosed until the whole unit matures and is shed from the plant (Meyerowitz, 1989). *Arabidopsis* is very useful in the study of dehiscent fruit (including canola, soybeans and lentils) because its silique (or seedpod) is representative of the fruit of over 3,000 species of the Brassicaceae family (Robles and Pelaz, 2005; Ferrandiz et al., 1999). It is a convenient specimen for experimentation because of its fast generation time (5-6 weeks) and its ability to flourish under a wide range of experimental conditions (e.g. growth temperatures, fluorescent or natural lighting, simple soils or specialized medias, etc). In addition, many plants may be grown in a confined space due to its small size and minimal growth requirements. *Arabidopsis* is also a valuable model in plant genetics, as its genome is relatively small,

consisting of five chromosomes, and several genetic maps and molecular markers have already been made available (Meyerowitz, 1989). The completion of the *Arabidopsis* genome sequencing project in 2000 was a major milestone in plant genomics, with the majority of its genes being identified and functionally classified (Immink and Angenent, 2002). Furthermore, the integration of Ti plasmids from *Agrobacterium* into the chromosomes of *Arabidopsis* has proven to be a simple yet effective method of expressing particular genes in a plants' offspring. With such a convenient method of gene transfer, along with the extensive resources available for mapping, it is no wonder the early botanist, R. O. Whyte, called *Arabidopsis* the "botanical Drosophila" (Meyerowitz, 1989).

Flower Development

Organ development in plants occurs in a similar process as limb development in animals – one system defines the type of organ to develop, while another defines the coordinates of that organ (Dinneny et al., 2005). Flowering plants, or angiosperms, start off as a mass of undifferentiated cells, known as the shoot apical meristem (Meyerowitz, 1989). There are two main phases of development – vegetative and reproductive – that are driven by various environmental and endogenous factors (Meyerowitz, 1989; Ferrandiz et al., 1999; Yamaguchi et al., 2009). The transition from vegetative to reproductive phases results in the transformation of the shoot apical meristem into an inflorescence meristem.

Floral meristems develop laterally from the inflorescence meristem, each containing whorled organ primordia (Ferrandiz et al., 1999) (Fig. 1). *Arabidopsis* contains four whorls, or rings, of organs. The outermost whorl consists of four sepals, followed by four petals in the adjacent inner whorl. Within the sepals and petals lies a ring of six stamens, the male reproductive organs, which surround the innermost whorl of carpels, or the female reproductive organs (Fig. 2). These four sets of organs comprise the flower, with the two fused carpels in the center making up the gynoecium, or fruit (Meyerowitz, 1989; Robles and Pelaz, 2005; Ferrandiz et al., 1999; Roeder and Yanofsky, 2006).

Once mature flowers have formed, fertilization may take place, initiating fruit development and the production of the ovules (or seeds) within the gynoecium (Ferrandiz et al., 1999; Roeder and Yanofsky, 2006). *Arabidopsis* gynoecia consist of many distinct cell types, with its outer morphology broken down into four main regions – from top to bottom (or apical to basal) they are the stigma, style, ovary and gynophore (Roeder and Yanofsky, 2006) (Fig. 3). The stigma, located at the apex of gynoecium, is composed of elongated epidermal cells that secrete a polysaccharide-rich extracellular matrix, which specializes in the adherence and germination of pollen grains. As the first component of the transmitting tract, the stigma plays a significant role in attracting pollen into the pollen tubes (Ferrandiz et al., 1999; Roeder and Yanofsky, 2006). Below the stigma lies the style – a short, solid cylinder that surrounds the transmitting tract. Its rectangular cells consist of wax ridges, or crenulations, and stomata. The style guides pollen from

the stigma to the ovary for fertilization of ovule primordia (Ferrandiz et al., 1999; Roeder and Yanofsky, 2006).

The ovary makes up the majority of the gynoecium and houses developing ovules (Roeder and Yanofsky, 2006) (Fig. 3). A strip of tissue, termed the septum, internally divides the ovary into two chambers, or locules, and is connected to the ovules via projections called funiculi (Robles and Pelaz, 2005; Ferrandiz et al., 1999; Roeder and Yanofsky, 2006). Beneath the ovary, at the very base of the gynoecium, is a short stalk known as the gynophore or internode (Fig. 3).

The ovary is the most complex structure within fruit, consisting of several discrete tissue types. Valve tissue makes up the walls of ovaries and is involved with the protection of ovules throughout fruit development, as well as the subsequent dispersal of mature seeds at dehiscence (Roeder and Yanofsky, 2006; Dinneny et al., 2005). Valve tissue is comprised of six layers of epidermal cells (Robles and Pelaz, 2005). The outermost (abaxial) layer, or exocarp, consists of long rectangular cells interspersed with stomata. Inside the exocarp, three layers of photosynthetic, chloroplast-containing cells (chlorenchyma cells) make up the mesocarp. The inner (adaxial) layer, or endocarp, consist of two cell layers termed en*a* and en*b*. The en*a* layer is the innermost layer of the two, and is composed of large, thin-walled cells, while the en*b* layer consists of narrow, elongated cells, that become lignified later in fruit development (Ferrandiz et al., 1999; Roeder and Yanofsky, 2006).

Two lateral valves are separated by a central ridge of tissue, called the replum, which contains a medial vascular bundle that extends throughout the lengths of ovaries

(Fig. 3). Repla, unlike valves, remain attached to the silique after pod shattering and the release of seeds (Robles and Pelaz, 2005; Dinneny et al., 2005).

A thin layer of cells connects valve and replum tissue, and is known as the valve margin or "dehiscence zone" (Roeder and Yanofsky, 2006). This specialized strip of tissue facilitates the detachment of valves from the replum during dehiscence and is composed of two cell types (Robles and Pelaz, 2005). The adaxial layer of valve margin tissue, or the "separation layer," borders either side of the replum and consists of small, isodiamteric cells. These cells secrete hydrolytic enzymes that disrupt cellular cohesion, thus creating a detachment line between the replum and valves (Robles and Pelaz, 2005; Roeder and Yanofsky, 2006) (Fig. 3). The abaxial valve margin layer, or "lignified layer," is adjacent to valve tissue and continuous with the en*b* layer of valve endocarp cells. The lignified layers of the valve and valve margin create tension along the dehiscence zone, driving the detachment of valve tissue from the replum (Robles and Pelaz, 2005; Roeder and Yanofsky, 2006; Dinneny et al., 2005). Once the fruit is opened, the simple mechanical force of wind, rain or physical contact will release seeds from the silique (Robles and Pelaz, 2005).

Genetic Factors Controlling Fruit Development

Proper development of the gynoecium requires the interaction of numerous genes from various different growth pathways, and their collective functions on regulating genes involved with fruit development. The complexity of this regulatory network is

what allows for the differentiation of distinct fruit tissues, as well as the specification of where these cells develop within the gynoecium. Although enormous progress has been made over the past few years in identifying key regulators in this genetic network, much is still unknown about the direct functions of, and relationships between, these genes. The distinction between individual functions and cooperative activities (with other transcription factors) remains largely unclear (Roeder and Yanofsky, 2006). However, the balance of these genetic regulators, along with the integration of environmental cues, is the backbone for generating fertile flowers capable of producing viable, seed-bearing fruit (Ciannamea et al., 2006).

Through a series of gain- and loss-of-function genetic studies, the main activities of several genes vital to the structuring of flowers have been uncovered. Single mutations causing prominent defects in floral patterning or the differentiation of tissues help direct genetic experiments towards identifying those genes and their individual functions. In this fashion, genes essential for replum, valve, and valve margin development have been discovered.

Valve margin cells will not differentiate without the activities of *SHP1*, *SHP2*, *IND*, and *ALC*. *INDEHESCENT* (*IND*) and *ALCATRAZ* (*ALC*) both encode for basic helix-loop-helix (bHLH) transcription factors, and are expressed in the valve margins of developing fruit. *ALC* is specifically involved with the differentiation of the separation layer of valve margins (layer adjacent to the replum), while *IND* plays a role in the development of both separation and lignified layers of the valve margin (Robles and Pelaz, 2005; Roeder and Yanofsky, 2006) (Fig. 4).

SHATTERPROOF 1 (*SHP1*) and *SHATTERPROOF 2* (*SHP2*) are two closely related MADS-box genes that redundantly promote the formation of valve margins (Robles and Pelaz, 2005; Roeder and Yanofsky, 2006). *SHP1,2* transcripts are detected early in carpel primordia, and later localize to the inner tissues of the gynoecium (i.e. the placenta, ovule primordia, septum, valve margins, and medial style vasculature) around stage 9 of flower development. Within the valve margin, they collectively activate *IND* and *ALC* in the production of the dehiscence zone (Fig. 4).

Misexpression of any one of these four valve margin genes causes a significant loss in valve margin development, often impacting other fruit structures in the process and rendering the fruit indehiscent. The precise patterning of lignified and separation layer cells between the valve and replum is key for successful pod shattering and the release of seeds. Thus, the regulation of valve margin genes is crucial for the overall fertility of the plant. Part of this regulation comes from the opposing activities of neighboring genes in the replum and valves, which repress valve margin genes from being expressed in surrounding tissues.

The MADS-box gene, *FRUITFULL* (*FUL*), is mainly involved with the development of valve tissue, which is made apparent in phenotypes of mutant *ful* fruit. In the absence of *FUL* activity, resulting fruit show a drastic reduction in ovary size, while the styles and gynophores are elongated (Robles and Pelaz, 2005; Roeder and Yanofsky, 2006). In addition to such defects in organ polarity, scanning electron micrographs reveal patches of compact, lignified cells in place of the long, slender cells of wild-type (WT) valves. It has since been established that *FUL* functions to represses valve margin

genes from being expressed in valve tissue (Fig. 4), thus responsible for preventing the ectopic lignification of ovary walls (Robles and Pelaz, 2005; Ferrandiz et al., 1999; Roeder and Yanofsky, 2006).

Similar to *FUL* in the valves, *REPLUMLESS* (*RPL*) negatively regulates the valve margin genes, *SHP*, *IND* and *ALC*, in the replum (Robles and Pelaz, 2005; Ferrandiz et al., 1999; Roeder and Yanofsky, 2006). *RPL* encodes a BELL-family homeodomain transcription factor that is involved in replum development. A loss of *RPL* activity results in the replacement of replum tissue with valve margin cells. Not surprisingly, *ful rpl* double mutants show ectopic valve margin identity in valve and replum tissue, covering the surface of ovaries in tiny, lignified cells. Thus, *FUL* and *RPL* function in parallel pathways to repress valve margin gene expression from expanding into the valves and replum, respectively, and restrict *SHP*, *IND* and *ALC* activities to their corresponding domains within the valve margin (Fig. 4).

The genes discussed above are necessary for proper development of *Arabidopsis* gynoecia, and function as immediate or "direct" regulators of valve, replum, and valve margin tissue. However, there are many upstream regulators of these genes that "indirectly" contribute to the production of these outer gynoecial tissues by interacting with such genes and influencing their activities. These interactions tend to be very complicated and involve a multitude of different factors, many of which have yet to be discovered. However, it is worth mentioning the activities of three particular regulators – *FIL*, *YAB3* and *JAG* – whose functions most directly influence *FUL*, *RPL*, *SHP*, *IND* and *ALC* activity and affect fruit morphology.

FILAMENTOUS FLOWER (*FIL*) and *YABBY3* (*YAB3*) are closely related *YABBY* genes that encode basic helix-loop-helix (bHLH) transcription factors containing one or more zinc finger. Both genes are expressed in the valves and valve margins of young carpels and are believed to be involved with regulating tissue polarity in lateral organs (Dinneny et al., 2005). Within developing gynoecia, they are important for patterning valve and valve margin cells and ensuring the lignification of appropriate cell layers. *fil yab3* double mutants produce highly deformed, indehiscent fruit – the apical region of *fil yab3* gynoecia lacks valve margin tissue or any kind of lignified cells, while the basal half develops ectopic valve margin identity with all valve and valve margin cell layers lignified. In addition, *SHP2* activity is absent in the apical region of *fil yab3* fruit, but ectopically expressed in valves and valve margins of basal fruit tissue. Furthermore, *FUL* activity was shown to be absent from all valve tissue, and could only be detected in style and gynophore cells. Thus, it is likely that *FIL* and *YAB3* positively regulate *FUL* and *SHP* in the valves and valve margins, respectively (Dinneny et al., 2005) (Fig. 4).

JAGGED (*JAG*), an unrelated gene encoding a C_2H_2 zinc finger transcription factor, is also involved with patterning tissues in lateral organs. It, too, is expressed in valve and valve margin domains, with redundant functions as *FIL* and *YAB3* in promoting *FUL* and *SHP* activity (Dinneny et al., 2005). When *JAG* and *FIL* activity is eliminated in *jag fil* double mutants, the resulting siliques (or fruit) have ectopic valve margin identity covering the surface of ovaries. As in *ful* mutants, the lengths of *jag fil* ovaries are reduced, while stylar and gynophore structures are elongated. The ectopic *SHP* and absence of *FUL* activity in *jag fil* valves further suggests that *JAG* functions analogously

with *FIL* and *YAB3* to regulate the boundaries of valve and valve margin domains within the gynoecium (Dinneny et al., 2005) (Fig. 4).

In addition to repressing valve margin genes in the replum, *RPL* negatively regulates *FIL* and *JAG*, dividing their activities to separate domains to create the two layers of the valve margin (Dinneny et al., 2005). While *FIL*, *YAB3* and *JAG* act upstream to promote *FUL* in valve development, *RPL* interacts with upstream class I *KNOTTED1*-like homeobox (*KNOX*) genes to promote replum development (Alonso-Cantabrana et al., 2007). RPL transcripts have the affinity to heterodimerize with KNOX transcription factors such as *BREVIPEDICELLUS* (*BP*), *KNAT2* and *KNAT6* (Fig. 4). These "replum factors" interact with RPL in the replum to support replum growth, while "valve factors" such as *FIL*, *YAB3* and *JAG* negatively regulate this group of *KNOX* genes in the valves and partially in the valve margins (Alonso-Cantabrana et al., 2007).

ASYMMETRIC LEAVES1 (*AS1*) and *ASYMMETRIC LEAVES2* (*AS2*), act as upstream regulators for the *KNOX* genes (Fig. 4). *AS1* (which produces an myb transcription factor) and *AS2* (whose transcripts contain a LATERAL ORGAN BOUNDARIES domain) are redundant genes involved in leaf differentiation. Both are highly active in valve tissue, where they help *FUL* repress valve margin gene activity by negatively regulating the *KNOX* genes. In summary, valve, replum, and valve margin tissues are specified by antagonistic factors (valve vs. replum), with valve margin tissue produced where the activities of valve and replum factors overlap (Alonso-Cantabrana et al., 2007).

FRUITFULL (FUL)

The key roles the aforementioned genes play in fruit are not only valuable for genetic and morphological plant studies, but also for their potential applications in farming culture. Extensive research is still being done to elucidate all pieces of this "molecular puzzle" and to discover how each piece fits together. In this paper, we take a closer look at *FUL* and how its expression is regulated to ensure proper valve development.

FUL is a member of the *AGL8* MADS-box gene family in *Arabidopsis* and is endogenously expressed early in plant development in the floral meristem. However, *FUL* expression becomes restricted to valve tissue by stage 8, with some expression also detected in the style, stems, leaves, and perianth (sepal and petal) vasculature (Ferrandiz et al., 1999). *ful* mutants are easy to detect, as their siliques are much shorter than wild-type and have strong aberrations in gynoecium morphology – they fail to develop stomata, valve mesocarp layers become ectopically lignified, styles are elongated, and repla adopt a "twisted" or "zigzag" shape down the medial ovary. *ful* mutant fruit are indehiscent, and ovaries often rupture prematurely due to built-up internal pressure, releasing smaller, underdeveloped seeds (Robles and Pelaz, 2005). Though much has been uncovered about *FUL*'s role in fruit patterning, the transcriptional regulation of *FUL* is still poorly understood. Information about transcription factors that influence *FUL* activity, or more specifically, individual promoter elements that are necessary for driving *FUL* transcription, is largely incomplete.

Previous *FUL* Research

In recent years, the Yanofsky lab has made great leaps towards answering such questions in their attempt to unravel the dense network of genetic pathways controlling fruit development. Much of the preliminary work necessary for such research was done by a former Master's student of the Yanofsky lab, Ann Nguyen (Nguyen, 2008). A minimal promoter region was identified upstream the *FUL* start codon, spanning roughly 3.9 kb in length (Fig. 5), and was sufficient in driving WT-like *FUL* expression in transgenic plants.

Through a series of 5' and 3' promoter deletions, different regions of the *FUL* promoter were found to drive *FUL* to be expressed in different tissues of the plant (Nguyen, 2008). For instance, 3' deletion constructs (i.e. constructs having various segments removed from the 3' end of the 3.9kb *FUL* promoter) demonstrated that motifs within the 3' promoter region are crucial for WT-like *FUL* expression in the inflorescence stem. While *FUL* is normally expressed throughout stem tissue up to the base of inflorescence buds, this expression faded as larger 3' deletions were made to the promoter (Nguyen, 2008). The largest 3' deletion construct was still capable of driving expression in valve and stylar tissue, though inflorescence stem expression was eliminated (Nguyen, 2008).

In addition, the 5' deletion constructs revealed that the 5' promoter region is involved with activating *FUL* activity in the valves, as well as repressing expression in the replum. When increasingly larger segments were removed from the 5' promoter end, reporter constructs showed little to no signal in the valves, style and sepal vasculature,

while activity in the inflorescence stem appeared unaffected (Nguyen, 2008). Furthermore, cross sections of these lines revealed abnormal activity in the replum, with ectopic expression detected in lateral replum tissue, in addition to the WT-like medial replum signal.

With a general idea of how different regions of the *FUL* promoter control different aspects of *FUL* function, it becomes much more manageable to identify specific promoter elements within these regions and delineate their individual roles in regulating *FUL*. The 3.9kb promoter region contains many regulatory elements that are currently being dissected. Nguyen's experiments set this off with a point mutation of a CArG-box motif, which suggested a possible role in repressing *FUL* expression in ovules (Nguyen, 2008).

FUL Promoter Elements

These findings set solid groundwork to further narrow down which promoter elements are responsible for driving certain expression patterns of *FUL* and, collectively, are necessary for proper ovule development and seed dispersal. The following preliminary experiments are based on the generation of reporter constructs driven by various fragments of the *FUL* promoter, allowing the visualization of different expression patterns that result from the action of individual motifs. There are five main groups of *FUL* transcription factors that have been selected for this comprehensive study. Previous research suggested strong potential for these factors in regulating *FUL* transcription, and

a schematic diagram of these elements and their positions relative to the *FUL* coding region can be seen in Figure 5. This methodical break-down of the *FUL* promoter region is the first step to understanding what drives *FUL* transcription, and ultimately, how *FUL* fits into the overall genetic network of fruit development.

MATERIALS AND METHODS

Cloning Strategy

The putative 3.9 kb *FUL* promoter, spanning from 66 bp to 3938 bp upstream the *FUL* start codon, was previously isolated and cloned into pDW294 and denoted pAN4 (Nguyen, 2007). This pAN4 construct was used a template in phusion PCRs to amplify and isolate various fragments of the *FUL* promoter flanked by restriction sites on each end (see Table 1 for list of oligonucleotides and their sequences). These fragments were subcloned into a PCR 2.1 TOPO vector (Invitrogen) or into a pGEMT vector (Promega) after 3' polyadenylation. To create GUS reporting lines, these promoter fragments were released via BamHI/PstI and cloned into the T-DNA vector, pDW294, which contains a minimal CaMV 35S promoter that drives the transcription of GUS (Hong et al., 2003). The integrity of each fragment and its joints to pDW294 was checked by digestion and sequencing.

Plant Transformation

The aforementioned GUS reporter constructs were transformed into *Agrobacterium tumefaciencs* (strain AGL0) via electroporation. They were then transformed into *Arabidopsis thaliana* plants (Col background) following the floral dip method (Clough and Ben, 1998). Plants expressing these GUS constructs were selected for on MS plates containing kanamycin.

GUS Staining

Tissue samples were first treated with 90% acetone for 15 min on ice, then washed with DI water for 15 min. They were then vacuum infiltrated with staining solution (25mM sodium phosphate, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 1% Triton X-100, 2mM X-Gluc) for 10 min at room temperature before being incubated overnight at 37°C (Alonso-Cantabrana et al., 2007).

Tissues were fixed in FAA (50% ethanol, 3.7% formaldehyde, 5% acetic acid) for 2.5 hours, then taken through an ethanol and Histoclear series before being embedded in Paraplast Plus.

Tissue sections were generated using a Jung Biocut (Leyca) microtome set to 8μm thick. A standard dissecting scope with an attached camera was used to take whole mount pictures of tissue samples. Slides were prepared and viewed as previously described (Roeder et al., 2003).

Site-directed mutagenesis

There are two auxin response elements (AuxREs) and two CArG-box motifs (Fig. 5) contained within the 3.9 kb *FUL* promoter, each of which were mutagenized to create GUS reporter constructs (Fig. 6). The four ARE and CArG motifs were individually mutagenized via PCR, using the corresponding sets of overlapping primers outlined in Table 2, and pAN4 (Nguyen, 2008) as the precursor. The PCR products were cloned into pGEM-T (as described earlier) to yield four preliminary constructs, each containing a single AuxRE or CArG mutation. For naming purposes, each pair of motifs was

distinguished as either 5’ (further from *FUL* coding region) or 3’ (closer to coding region), and mutation constructs were labeled: AuxRE -/+ (5’ AuxRE mutation), AuxRE +/- (3’ AuxRE mutation), CArG -/+ (5’ CArG mutation) and CArG +/- (3’ CArG mutation).

Promoters containing more than one mutation were synthesized by combining fragments of the preliminary constructs listed above. AuxRE -/- (both AuxRE motifs mutated) constructs were made by replacing the (WT) 3’ AuxRE region in AuxRE -/+ constructs with a (mutated) 3’ AuxRE region from ARE +/- constructs via NsiI/BamHI. Likewise, CArG -/- constructs were made by replacing the (WT) 3’ CArG region in CArG -/+ (or pAN26 from Nguyen, 2008) constructs with the mutated region in CArG +/- constructs via BstBI/BamHI. The central promoter region, containing both CArG mutations, was excised from CArG -/- and inserted into AuxRE -/- constructs, via NdeI/NsiI, to create the AuxRE -/- CArG -/- constructs which have all four AuxRE and CArG promoter elements mutated.

Each of the mutagenized promoter fragments were extracted from their pGEM-T vectors after sequencing, and shuttled into pDW (via BamHI) to create seven distinct reporter constructs (Fig.6). All oligonucleotides and restriction enzymes used to create and verify these constructs are included in Table 2, with the mutated sequences indicated.

RESULTS

As mentioned earlier, prior studies on the regulation of *FUL* transcription were carried out by a former graduate student in Prof. Martin F. Yanofsky's laboratory (Nguyen, 2008). The putative 3.9 kb regulatory region (or promoter) was sufficient in driving wild type-like *FUL* expression, mimicking endogenous *FUL* expression patterns previously characterized (Gu et al., 1998; Ferrándiz et al., 2000; Nguyen, 2008). A series of *FUL* "promoter bashing" experiments were performed in which various fragments of the 3.9 kb *FUL* promoter were used to drive *GUS* activity in reporter constructs, and the resulting expression patterns were analyzed for information regarding *FUL* regulation. From these preliminary studies, it was deduced that the 5' promoter region is involved with controlling *FUL* transcription in the valves, while the 3' region is important for early flower development and inflorescence stem expression. In addition, it was suggested that a 5' CArG-box motif (located 2512 bp upstream the *FUL* start codon) is involved with repressing *FUL* in developing ovules (Nguyen, 2008, our unpublished data).

These early findings have paved the way for further investigation, such as the research presented here, which aims to better our understanding of *FUL* transcriptional regulation. By isolating the effects that specific promoter elements have on *FUL* expression (via *GUS* reporter lines), we may more accurately place *FUL* within the overall genetic network responsible for directing fruit development. Though the following research barely skims the surface of the vast sea of pathways and interactions, we have succeeded in identifying portions of the *FUL* promoter that specifically regulate *FUL* activity in the valves.

Analysis of Motifs Found In *FUL* Promoter Region

From PLACE analysis of the 3.9 kb *FUL* promoter, as well as the "regional" regulatory data available from previous studies (Nguyen, 2008), we selected seven putative promoter elements (outlined in Fig. 5B) that may play significant roles in regulating *FUL* activity, especially *FUL*'s function in valve development. In this section, we present the results obtained from experiments analyzing such motifs.

Part I. CArG-Box Regulation of *FUL* Transcription in Flowers

A very common family of transcription factors in plants is the MADS-box family of proteins, which bind specific promoter sequences upstream their target genes to positively regulate transcription. The DNA-binding site recognized by MADS-box factors is known as the "CArG-box," and has the conserved consensus sequence, $CC(A/T)_6GG$ (Kamada and Miwa, 1992; Ciannamea et al., 2006). There are two perfect CArG-boxes located within the 3.9 kb *FUL* promoter – one 2038 bp upstream *FUL*'s start codon (referred to as the 3' CArG) and the other 2512 bp upstream (5' CArG) (Fig. 5B).

MADS-box transcription factors and CArG-boxes are common among many different species in addition to plants, such as fungi, yeast and animals (Masiero et al., 2002). The MADS-box genes in the *Arabidopsis thaliana* genome play key regulatory roles in several aspects of development, such as controlling flowering time, phase transitions, root development, inflorescence and floral meristem identity, determination of organ fate, fruit patterning (the case of *FUL*), and dehiscence (Roeder and Yanofsky, 2006).

The various MADS-box proteins coded for by this group of genes all share similar secondary structures. This structure consists of the following four domains (in order from N-to-C termini): a highly conserved MADS-box (M) that functions as the DNA-binding domain, followed by the intervening region (I) and coiled-coil keratin-like region (K) which collectively assist in protein-protein interactions and dimerization, and finally, the C-terminus domain (C) which makes up the most variable region of MADS-box proteins and believed to enhance the selectivity of target genes for regulation (Masiero et al., 2002). These MADS proteins are also called MIKC proteins due to their domains.

FUL is a member of this MADS-box gene family and likely interacts with other MADS factors to carry out its role in the development of valve tissue. *FUL*'s critical role in valve development is crucial for overall fruit morphogenesis, including ovule development and proper dehiscence to release seeds. Thus, it is essential to maintain strict regulation of *FUL*, and a set of transcription factors (or promoter motifs) interact upstream *FUL*'s coding region to activate its transcription. The two CArG-boxes located within *FUL*'s 3.9 kb promoter are good candidates for such transcriptional regulation, though *how* they affect *FUL* transcription is still unknown. Are they involved in the *spatial* regulation of *FUL*, such as the apical/basal gradient seen in valve tissue after anthesis, or are they more involved with *temporal* regulation, such as determining when to activate *FUL* throughout vegetative and reproductive growth phases?

To answer these questions, we generated transgenic lines harboring one or more CArG-box mutations that are efficient in preventing the binding of MADS proteins. By analyzing the resulting loss-of-function phenotypes, we may gain knowledge of how the

CArG motifs affect *FUL* transcription (Masiero et al., 2002). Three *GUS* reporter constructs were created, each having different CArG-box mutations within the 3.9 kb *FUL* promoter – these fragments were cloned into pDW vectors to drive *GUS* expression (Fig. 6). These constructs were then used to transform *Arabidopsis thaliana* plants (Colombia background), with the name of each transgenic line indicating which CArG-box has been mutated – the sign on the left refers to the 5' CArG-box, while the one on the right refers to the 3' CArG ("-" = mutation; "+" = WT). Thus, the three resulting lines are as follows: *CArG-/+::GUS* (for those having a mutated 5' CArG-box), *CArG+/-::GUS* (those with a 3' CArG mutation), and *CArG-/-::GUS* (having both CArG-boxes mutated). GUS staining of these transgenic lines indicate where *FUL* is being expressed, and reveal how the particular promoter motifs present regulate *FUL* transcription.

5' CArG Mutation (CArG -/+)

The 5' CArG-box – located further upstream *FUL*'s coding region at -2512 bp – was mutated in previous studies and cloned into pDW to create *CArG -/+::GUS* constructs (Nguyen, 2008). The *GUS* expression in these lines displayed similar expression patterns to those of the WT *FUL* control (pDW lines in which *GUS* expression is driven by the WT 3.9kb *FUL* promoter fragment, or *FUL::GUS* (Fig. 7C).

CArG -/+::GUS plants showed *GUS* signal in the inflorescence stem, and fruits displayed an apical/basal expression gradient in valve tissue. Cross sections of *CArG -/+ ::GUS* fruit, however, revealed ectopic replum expression (in contrast to the strict medial replum domain of WT *FUL*) and scattered signal throughout the ovule primordia (no expression at all in WT *FUL*) (Fig. 8A,C). This suggests that the 5' CArG-box may be

involved with repressing *FUL* transcription in lateral replum tissue and ovule primordia, acting as a negative regulator of *FUL*.

3' CArG Mutation (CArG +/-)

In addition to the 5' CArG-box, *FUL*'s promoter also contains a 3' CArG-box, positioned slightly closer to the start codon at -2038 bp (Fig. 5). To see whether this CArG motif functions in a similar fashion as the 5' CArG, *CArG +/-::GUS* lines were generated, having a 3' CArG-box mutation.

The whole mount expression patterns for these lines resembled those of WT *FUL::GUS*, with *GUS* signal detected early in the floral meristem before being localized to valve tissue by stage 8 of flower development. After anthesis (stage 12), however, deviations in expression patterns become more apparent. In WT *FUL::GUS* gynoecia, valve expression begins to concentrate in the apical (style-proximal) and basal (gynophore-proximal) regions, while medial valve expression levels fade (Fig. 7B). This apical/basal or "bipolar" gradient of *FUL* activity is abolished in *CArG +/-::GUS* lines, which portray high expression levels throughout the lengths of valve tissue (Fig. 7D). In addition to the irregular valve patterning, these lines also showed reduced signal in the style (Fig. 7B,D).

Cross sections of *CArG +/- ::GUS* fruit exposed similar abnormalities as CArG -/+::*GUS* fruit, though to a slightly lesser degree. Ectopic expression is detected in the lateral replum tissue of young *CArG +/- ::GUS* carpels, though this expression vanishes by stage 14 of flower development (Fig. 8E,F). Since the loss-of-function phenotype for *CArG +/- ::GUS* lines involved an increase in active *FUL* domains (i.e. lateral replum

and medial valve tissue), the 3' CArG-box likely acts as a negative regulator of *FUL* transcription, similar to the upstream 5' CArG motif.

Double CArG Mutation (CArG -/-)

The third and final CArG construct created, *CArG -/-::GUS*, contains mutations of both the 5' and 3' CArG-boxes in *FUL*'s promoter. Since each CArG-box motif binds to an MIKC (MADS-box) protein, and MIKC transcription factors contain several domains specialized in protein-protein interactions, it is likely these MIKC proteins are binding other similar transcription factors in addition to their respective CArG consensus sequences. By dimerizing with other CArG-bound transcription factors, they form heterodimer complexes that are better suited to regulate transcription. The loss-of-function phenotype of *CArG -/- ::GUS* lines may illuminate some of these more complex regulatory pathways and indicate whether the two CArG-boxes interact to repress *FUL* transcription.

Interestingly, when all CArG-box binding is eliminated in *CArG -/- ::GUS* lines, virtually no *FUL* transcription occurs throughout the plant (Fig. 7E). Cross sections of *CArG -/-::GUS* fruit failed to show even faint signal in valve cells (Fig. 8G,H). Unlike the single CArG mutations that resulted in additional replum and ovule expression, the double CArG-box mutation had an opposite effect on *FUL* transcription – not only was there no ectopic fruit expression, but the tissues in which *FUL* is normally expressed were lacking signal as well (i.e. valve tissue).

It was not until cross sections of whole *CArG -/- ::GUS* inflorescences were examined before any *GUS* signal could be detected in these transgenic lines. This

broadened view revealed high *GUS* signal in the pollen sacs of stamens, where *FUL* is not normally expressed (data not shown).

While the loss of a single CArG-box does not greatly affect *FUL* transcription as portrayed by the *CArG -/+::GUS* and *CArG +/-::GUS* lines, at least one of the two CArG motifs must be functioning in order for any kind of *FUL* transcription to occur.

Part II. Roles of Auxin Response Elements (AuxREs) in Regulating *FUL* Transcription

There are also two canonical Auxin Response Elements (AuxREs) located within the 3.9 kb *FUL* promoter, each having the consensus sequence TGTCTC (Ulmasov et al., 1995). The two auxin-responsive promoter elements are positioned about 3 kb apart – one is located 3,679 bp upstream, while the other is only 641 bp upstream, directly preceding *FUL*'s start codon. As with the naming of the two CArG-box motifs, the two AuxRE's are distinguished as the 5' AuxRE (-3,679 bp) and the 3' AuxRE (-641 bp) (Fig. 5B).

Auxin Response Factors (ARFs)

Auxin Response Factors (ARFs) are transcription factors that bind specifically to AuxRE motifs, and may positively or negatively regulate the expression of auxin response genes (Liscum and Reed, 2002; Guilfoyle and Hagen, 2007). Since auxin is such an integral plant hormone involved in a variety of developmental processes, it is likely that auxin-response factors (ARFs) are influencing *FUL* activity in valve and fruit development. Auxin/indole acetic acid (Aux/IAA) repressors have been shown to

dimerize with ARFs, and negatively influence their regulation of target genes, leading to a decrease in auxin-induced gene expression (Abel et al., 1994, 1995; Ulmasov et al., 1997). Thus, it is possible that the AuxREs are acting upstream to repress *FUL* transcription in response to auxin.

While we may expect the two AuxREs are involved with negative regulation, it is unknown which ARFs interact with them to influence *FUL* transcription. Thus, we generated three *GUS*-reporter constructs in which one or both of the AuxREs were mutated, similar to the CArG constructs. The resulting transgenic lines were denoted: *AuxRE -/+:: GUS* (5' AuxRE mutation), *AuxRE +/-::GUS* (3' AuxRE mutation), and *CArG -/-::GUS* (mutations of both AuxREs).

5' AuxRE Mutation (AuxRE -/+)

To generate *AuxRE-/+::GUS* reporter lines, the 5' AuxRE consensus sequence was changed from TGTCTC to TTGTCT (see Materials & Methods). Although the expression patterns of these transgenic plants mimicked those of WT *FUL::GUS*, the expression levels that were observed were much higher. Young *AuxRE -/+::GUS* plants displayed strong *GUS* signal in floral tissues, such as the carpel primordia and sepal vasculature. After anthesis, *AuxRE -/+::GUS* fruit retained a WT-like "bipolar gradient" throughout valve tissue, as well as strong style expression at the apex. In addition to the increased transcription levels, another notable discrepancy between the *AuxRE -/+::GUS* and WT *FUL::GUS* lines was that the former lacked inflorescence meristem activity (Fig. 9BC). Though some expression is visible in the stems, this expression fades as it approaches the junction of developing buds, where floral meristems branch out from the

inflorescence meristem (Fig. 9B,C).

The 5’ AuxRE loss-of-function phenotype was even more drastic within the inner gynoecial structures. Cross sections of younger *ARE -/+ ::GUS* fruit (before stage 14) revealed remarkable expression patterns. Instead of *FUL* activity being limited to valve and style tissues, as in the *FUL::GUS* WT controls, *AuxRE -/+::GUS* fruit showed strong signal in the septum, ovules and (lateral and medial) replum tissues (Fig. 10). And, as with the whole mount comparisons, the *levels* of this ectopic expression were higher than those seen in WT *FUL::GUS* cross sections. After stage 15, however, *AuxRE -/+::GUS* fruit begin to lose this excess signal and start resembling WT fruit expression patterns (Fig. 10C,F).

In conclusion, it appears that 5’ AuxRE’s most significant role is in negatively regulating the *levels* of *FUL* transcription, with its loss-of-function resulting in an overall increase in expression levels. The 5’ AuxRE motif is also involved with preventing *FUL* from being ectopically expressed in the gynoecium (e.g. septum, ovules, lateral replum). In addition to this negative regulation of *FUL*, the 5’ AuxRE may also act as a positive regulator early in development, activating *FUL* in the inflorescence meristem.

3’ AuxRE Mutation (AuxRE +/-)

As with the *AuxRE -/+::GUS* lines, *AuxRE +/- ::GUS* whole mounts portrayed WT-like expression patterns, though the levels of expression were increased (Fig. 9). However, instead of lacking inflorescence meristem signal like *AuxRE -/+::GUS* plants, *AuxRE +/- ::GUS* lines lack sepal vasculature expression (Fig. 9).

Furthermore, *AuxRE +/- ::GUS* fruit show aberrant expression patterns later in flower development. Young ovaries display a strong, uniform signal throughout the lengths of valve tissue (Fig. 9D). This solid expression, however, fails to evolve into the WT-like bipolar gradient as the fruit matures. After anthesis, valve expression is reduced and becomes blotchy, lacking any distinguishable pattern (Fig. 9B,D).

Analysis of *AuxRE +/- ::GUS* cross sections yielded similar results as *AuxRE -/+::GUS* in that *AuxRE+/-::GUS* gynoecia displayed ectopic signal in ovules, septum and replum tissue up to stage 15 of flower development (Fig. 10).

In conclusion, the 3' AuxRE motif likely serves to repress *levels* of *FUL* transcription along with the 5' AuxRE 3 kb upstream. The two AuxRE motifs also appear to redundantly repress *FUL* transcription in ovules, septum and lateral replum tissue. Furthermore, the 3' AuxRE motif may be involved with activating *FUL* in sepal vasculature, as its mutation led to a loss of expression here. Most interesting, however, is the possible role 3' AuxRE has in *patterning FUL* in valve tissue. Its loss-of-function resulted in irregular valve signal (i.e. no bipolar expression gradient), suggesting a role in spatial regulation of *FUL* transcription.

Double AuxRE Mutation (AuxRE -/-)

When both AuxREs were mutated in *AuxRE -/-::GUS* lines, multiple expression patterns result. Whole plants displayed WT-like *GUS* signal, with some lacking inflorescence meristem or sepal vasculature expression (Fig. 9E top vs. bot). As young carpel primordia develop into fruit, *GUS* signal becomes restricted to the valves of gynoecia, as in WT *FUL::GUS*. However, after fertilization, some *AuxRE -/- ::GUS* fruit

retained a solid expression gradient throughout valve tissue while others showed a localization of signal to apical and basal regions, more closely resembling the bipolar gradient seen in WT *FUL::GUS* controls (Fig. 9E). In addition, *AuxRE -/-::GUS* lines produced significantly higher *levels* of *GUS* expression than those seen in *FUL::GUS*, in congruency with the single-AuxRE-mutation lines.

Surprisingly, cross sections of *AuxRE -/- ::GUS* fruit produced much less "ectopic" *GUS* signal (i.e. in lateral replum cells, ovules and the septum) than the single-AuxRE-mutation lines. Though young *AuxRE -/-::GUS* gynoecia showed some *GUS* activity in lateral replum cells, there was very minimal expression detected in septums and ovules (Fig. 10J,K). By stage 15, all ectopic expression was eliminated from the septum and ovules, making *AuxRE -/- ::GUS* cross sections appear WT-like with only valve expression remaining (Fig. 10L).

Overall, mutating both AuxRE motifs did not have any novel regulatory effects on *FUL* transcription compared to the single AuxRE mutations. While both AuxRE's function redundantly in many respects (i.e. to repress *FUL* in the inner tissues of the gynoecium, as well as controlling overall transcription levels) each has subtle differences on *FUL* regulation (i.e. 5' ARE affects *FUL* transcription in the inflorescence meristem, while 3' AuxRE affects transcription in sepal vasculature). However, analysis of *AuxRE -/- ::GUS* lines suggests these motifs act individually, yet in an additive fashion, to influence *FUL* transcription. Double AuxRE mutants portrayed the loss-of-function phenotypes of both sets of single-AuxRE-mutation lines. Since the removal of AuxRE function results in an overall increase in transcription levels, we predict that certain ARFs are binding these AuxRE motifs to negatively regulate *FUL* transcriptional activity.

Quadruple AuxRE & CArG Mutations (AuxRE -/- , CArG -/-)

As explained earlier, the two CArG-boxes in *FUL*'s promoter most likely function as positive regulators, with little to no transcription occurring in double CArG mutants. Conversely, our results from the *AuxRE::GUS* lines suggest that the two AuxRE motifs function primarily as negative regulators of *FUL*. Since the CArG and AuxRE motifs have opposing effects on *FUL* transcription, it would be interesting to see what happens when all four motifs are mutated in *FUL*'s promoter. We generated a quadruple-mutation reporter line in which both AuxRE and both CArG-box motifs were mutated (denoted *AuxRE -/-, CArG -/- ::GUS*) (Fig. 6) to determine the strength of each motif's influence on *FUL* transcription in relation to one another.

These transgenic lines showed no *GUS* staining anywhere in the plant (figure not shown) – even cross sections of *AuxRE -/- CArG -/- ::GUS* fruit failed to reveal any signal within inner gynoecial tissues. These results are consistent with our findings from the CArG -/- constructs, which also failed to produce any *GUS* signal (Figs. 7,8). While single and double AuxRE mutations resulted in overall increases in transcription, the CArG mutations take precedence in *AuxRE -/- CArG -/- ::GUS* lines, preventing any transcription from being initiated. Thus, at least one CArG-box must be present for *FUL* transcription to occur, and the CArG-box motifs are epistatic to AuxRE motifs in regulating *FUL* activity.

Part III. "ABCD" Promoter Region Necessary for Valve-Specific Expression

While *FUL* is involved with several aspects of fruit development, its most significant role is ensuring the proper development of valve tissue. *FUL* activity is required in the valves to repress adjacent valve margin and replum genes from being expressed there. Thus, identifying specific motifs upstream the *FUL* coding region that are necessary for activating *FUL* transcription in the valves would be critical for understanding *FUL* regulation. In addition, the determination of such "valve-specific" promoter elements may shed light on candidate transcription factors that bind these motifs to regulate *FUL* in the valves. Uncovering these transcription factors and their corresponding genes will reveal some key upstream regulators of *FUL* and help fill in the overall genetic network controlling flower development.

Previous Findings on Valve-Specific Activation of *FUL*

Previous promoter bashing experiments have set the groundwork for such "valve-specific" studies and have succeeded in narrowing down a 682 bp promoter region, approximately 2.5 kb upstream *FUL* ATG (-2952 bp, -2271 bp), that was critical for *FUL* expression in valves (Nguyen, 2008). Nguyen, a former graduate student of the Yanofsky lab, began a series of "*FUL* promoter bashing" experiments, in which reporter constructs were created with various fragments of the *FUL* promoter region used to drive *GUS* expression. A control line was created with the WT 3.9 kb *FUL* promoter (-3938, -66) ligated into pDW294 plasmids (*GUS* vectors) – these transgenic lines displayed WT-like *GUS* signal, with fruit portraying the bipolar expression gradient in valve tissue after anthesis (Nguyen, 2008).

Several other reporter lines were generated from various fragments of the *FUL* promoter. Increasingly larger fragments were removed from the 5' promoter end, to observe which had an effect on *GUS* expression. The same was done to the 3' end of the promoter, showing that this region affected *FUL* transcription in the inflorescence meristem early in flower development (Nguyen, 2008).

However, when focusing on *FUL* expression in the valves (the unique bipolar gradient, in particular), Nguyen found a central promoter region that appeared to be crucial. The 682 bp region (-2952, -2271) was delineated from the smallest 5' and 3' deletion constructs still capable of driving WT-like *GUS* signal in the valves.

The "ABCD" *FUL* Promoter and Related Reporter Lines

To find putative motifs within *FUL*'s promoter that are responsible for regulating its transcription in valve tissue, we performed a PLACE analysis in the "valve-specific" vicinity previously determined. Three candidate motifs were selected for further investigation: MYB (located 2686 bp upstream *FUL* ATG, or at -2686), SAUR (-2839), and SBP (-2971) (Fig. 5). To isolate and highlight the roles these motifs play in regulating *FUL* expression in the valves, reporter constructs were created. Only the portion of the 3.9 kb *FUL* promoter containing these motifs was used to drive *GUS* expression in transgenic plants.

However, because it was previously discovered that at least one of the two CArG-box motifs in *FUL*'s promoter must be functional for *FUL* transcription to be initiated (as proven by the lack of signal seen in the *CArG -/- ::GUS* lines), at least one CArG-box must be present in the truncated "valve-specific" *FUL* promoter to drive any kind of *GUS*

expression in the resulting lines. Since the 5' CArG-box, located at -2512, is closer to the other three motifs than the 3' CArG-box (-2038), it too was included in each of the "valve-specific" constructs. The corresponding "valve-specific" transgenic lines will show how these motifs influence *FUL* transcription in the valves, and may determine the minimum promoter fragment necessary for driving WT-like *FUL* expression in the valves.

One "valve-specific" line, denoted *ABCD::GUS*, has *GUS* expression driven by all four motifs – 5' CArG-box, MYB, SAUR and SBP (-2971, -2512) (Fig. 6). Each letter, *A – D*, represents a single motif, with *A* = 5' CArG-box, *B* = MYB, *C* = SAUR, and *D* = SBP. Analyzing *ABCD::GUS* lines will show whether these four motifs, by themselves, are capable of driving *GUS* (or *FUL*) expression in the valves. If *ABCD::GUS* lines display *GUS* signal in the valves, especially the bipolar expression gradient seen in WT *FUL*, this will be the shortest promoter fragment shown to do so.

In addition, *ABC::GUS* lines were created that were identical to the *ABCD::GUS* lines except they lack the 5'-most SBP (*D*) motif (Fig. 6). The *GUS* expression patterns seen in the valves of these lines will show whether the MYB and SAUR motifs (along with the 5' CArG-box) are sufficient in driving *FUL* in the valves. Comparing these results with those of the *ABCD::GUS* lines will highlight the significance of the SBP motif in regulating *FUL* transcription in the valves.

ABCD::GUS Transgenic Lines Display High Reporter Expression in Valves

A 612 bp fragment from the 3.9 kb *FUL* promoter (-2971 bp, -2512 bp) was isolated and cloned into pDW294 vectors to generate the *ABCD::GUS* reporter constructs

(Fig. 6). Each of the four letters represents an individual promoter element, as follows, starting with the closest to *FUL*'s coding region (3' to 5'): 5'CArG-box ("A"), MYB ("B"), SAUR ("C"), and SBP ("D"). *ABCD::GUS* transgenic lines will thus test where these four motifs are capable of driving *FUL* transcription, and possibly defining the minimal promoter region necessary for regulating *FUL* expression in the valves.

The resulting *ABCD::GUS* lines did, in fact, exhibit strong *GUS* signal throughout valve tissue, though no signal was detected in the sepal vasculature or inflorescence stems, as observed in the WT *FUL::GUS* lines (Fig. 11C). *ABCD::GUS* gynoecia showed high levels of GUS activity throughout the lengths of valves and medial replum tissue, while sepals and stems displayed no signal (Fig. 11C). Despite the lack of an apical/basal expression gradient in the valves, this is the smallest promoter fragment yet capable of in driving "valve-specific" expression of *FUL*. Cross sections of *ABCD::GUS* gynoecia further resemble WT-like expression patterns, with high levels of *GUS* expressed in valve cells but no ectopic signal detected in other tissues, such as the ovule primordia, septum, and lateral replum (Fig. 12). When comparing the whole mount gynoecia from WT *FUL::GUS* and *ABCD::GUS* lines, it is apparent that both the expression *domains* and *levels* are higher in *ABCD::GUS* valves. *ABCD::GUS* valves are uniformly stained from style to gynophore (Fig. 11C), whereas *FUL::GUS* valves have an apical/basal gradient, leaving medial valve tissue unstained (Fig. 11B). The *levels* of GUS stain also appear darker in *ABCD::GUS* valves relative to *FUL::GUS*, and is especially noticeable in younger gynoecia, prior to fertilization (Fig.'s 11, 12).

ABC::GUS Transgenic Lines Exhibit Significantly Reduced Reporter Expression

To test whether the 612 bp "ABCD" promoter fragment used in *ABCD::GUS* constructs could be further reduced to drive valve-specific transcription, an additional 119 bp was removed from the 5' end. The resulting 493 bp promoter fragment (-2893 bp, -2512 bp), containing the same promoter elements as *ABCD::GUS* constructs with the exception of the SBP motif (or "D"), was cloned into pDW294 plasmids to create *ABC::GUS* constructs (Fig. 6). Though *ABC::GUS* lines were able to drive a similar valve-specific expression pattern as the *ABCD::GUS* lines, the transcription *levels* were drastically reduced (Fig. 11D). No reporter signal was detected in the inflorescence stem or sepal vasculature, similar to the SBP-containing *ABCD::GUS* lines. This suggests that the SBP motif ("D") plays a significant role in activating *FUL* transcription specifically in valve tissue. While the SBP motif may be important in controlling the *levels* of *FUL* expression in valves, it is not sufficient for creating the WT-like bipolar gradient seen in *FUL::GUS* lines (Fig. 11B). The cross sections of *ABC::GUS* fruit fail to show *GUS* expression in any gynoecial tissues except for valves (Fig. 12), further emphasizing the valve-specific expression pattern. In addition, almost all valve expression was lost by stage 15 in *ABC::GUS* fruit (Fig's 11, 12).

Overall, these results support those of the *ABCD::GUS* lines and confirms that the minimal "valve-specific" promoter fragment necessary to drive *FUL* in the valves is (-2952 bp, -2271 bp). Therefore, we conclude that the four motifs contained within *ABCD::GUS* constructs (i.e., the 5'CArG-box, MYB, SAUR and SBP motifs) are essential for directing *FUL*-related valve development, with the SBP motif playing a particularly significant role in regulating *FUL* transcription *levels* in the valves.

DISCUSSION

Part I. Regulatory Roles of CArG-Box Motifs in *FUL* Transcription

MADS-box Genes

MADS-box genes are present in many different species and play a variety of roles regulating growth and development. There are over 100 different MADS-box genes found in the *Arabidopsis thaliana* genome, and are involved in a variety of processes governing the transition between vegetative and reproductive developmental phases (Folter et al., 2005). Their functions range from determining primordial organ identity early in development, to mediating cell differentiation, division, and elongation in later stages. These genes act by positively or negatively regulating the expression of downstream target genes involved in flower development (Gan et al., 2005).

MADS-box genes are divided into many different classes, some with overlapping functions, but all communicate and work together in a unified regulatory network. The organization of this dense network rests upon a hierarchy of genetic relationships influenced by environmental factors, temporal and spatial cues, and the proximity of and/or interaction with other transcription factors (Gan et al., 2005; Folter et al., 2005).

The broad family of MADS genes can generally be categorized as either meristem identity genes or organ identity genes. Meristem identity genes regulate the development of meristematic structures, such as the transition from the shoot apical meristem (SAM) into an inflorescence meristem, or from the inflorescence meristem into a floral meristem (Theissen et al., 2000). Examples of such genes in *Arabidopsis* include *AP7*, *CAL*, and

LFY (Rounsley et al., 1995). Conversely, organ identity MADS-box genes regulate floral organ fate and determine the identity of the four major floral organs. MADS genes that fall under this class are also referred to as homeotic selectors, or "ABC genes" (in reference to the popular "ABC" model of flower development) (Urbanus et al., 2009). Floral organ identity genes can thus be further divided into three subclasses – "A", "B" or "C" – depending on the organs they effect. Mutations in class A genes affect sepal and petal identity in the first two whorls, while mutations in class B genes affect petals and stamens in the second and third organ whorls. Mutations in class C genes affect the reproductive organs, or the stamens and carpels in the innermost whorls (Theissen et al., 2000; Tani et al., 2009). These are all examples of homeotic mutations in which the identity of one or more whorls of organs is replaced by another, giving each subclass a distinct mutant phenotype. For example, *ap1* mutants (loss of class A functioning gene) develop carpels in place of sepals in the first whorl, and stamens in place of petals in the second (Mandel and Yanofsky, 1995).

MADS-box Transcription Factors

All MADS genes encode proteins that share a conserved a DNA-binding domain, or MADS domain. This 56-amino acid segment specifically binds to CArG-box motifs contained within target genes to positively or negatively regulate their transcription. In addition, MADS proteins may recruit the binding of nearby transcription factors, including other CArG-bound MADS proteins. The resulting tetrameric complexes influence gene transcription in different ways, and create compound levels of regulation (Riechmann and Meyerowitz, 1997).

To better understand how these MADS-box transcription factors bind specific genes to affect flower development, three additional major domains of MADS proteins will be discussed. Though the MADS domain is involved in DNA-binding, the *selectivity* with which these MADS proteins bind specific sequences of DNA is reliant on two adjacent domains, positioned immediately to the carboxyl side of the MADS domain, called the "I" and "K" domains. These domains serve more as dimerization platforms that influence the transcription factor's DNA-binding kinetics (Theissen et al., 2000; Mandel and Yanofsky, 1995). Since all MADS proteins have similar structures (MIKC domains) and bind the same DNA sequences (CArG-box motifs), the complex dimerization amongst multiple domains in MADS proteins is crucial for the transcription factors' selective regulatory potentials, and allows many closely-related MADS factors to carry out a variety of distinct functions. This functional specificity is what drives the radically changing expression patterns of homeotic genes throughout flower development (Shore and Sharrocks, 1995; Davies et al., 1996).

AGAMOUS, a class "C" organ identity MADS-box gene

AGAMOUS (*AG*) is a MADS-box gene involved in reproductive organ identity (i.e. the production of stamens and carpels). Early in flower development, AG RNA is present throughout the third and fourth whorls of the floral primordium, while later on, its expression becomes localized to specific cell layers of developing stamens and carpels (Bowman et al., 1991). *AG* falls under class "C" of the floral homeotic genes, with *ag* mutants having stamens replaced by petals in the third whorl, while carpels in the fourth whorl are replaced by an indeterminate pattern of perianth organs. When *AG* is

overexpressed (driven by *35S* promoter), mutants show sepal-to-carpel and petal-to-stamen conversions in the first and second organ whorls, respectively, yielding flowers consisting only of reproductive organs (Rutledge et al., 1998; Bowman et al., 1991).

APETALA1, a class "A" organ identity MADS-box gene

APETALA1 (*AP1*) is an organ identity MADS-box gene involved in the development of sepals and petals in the first two whorls of floral organs, thus making *AP1* a class "A" homeotic selector. Early in development, AP1 RNA uniformly accumulates throughout young floral primordia, while its expression becomes limited to the first and second whorls later on (Gustafson-Brown, 1994). As mentioned earlier, *ap* mutants have sepal-to-carpel and peteal-to-stamen conversions in the first and second whorls, respectively, yielding flowers consisting only of reproductive organs. Its overexpression in *35S::AP1* mutants, on the other hand, causes stamens to be replaced by petals in the third whorl and carpels to be replaced by sepals in the fourth (Mandel and Yanofsky, 1995).

Both AP1 and AG MADS-box transcription factors have been shown to interact with *FUL*, though in opposing ways, to regulate transcription and influence *FUL*'s unique expression pattern throughout flower development. *AP1* acts as a negative regulator and represses *FUL* activity in first two whorls of young floral primordia (i.e., the sepals and petals) early in flower development (Mandel and Yanofsky, 1995). Conversely, *AG* functions as a positive regulator of *FUL* via the repression of *AP1* after stage 3 of flower development. By inhibiting *AP1* repression of *FUL*, *AG* allows *FUL* activity to be initiated in the floral primordia, where it is eventually localized to fourth whorl

(Gustafson-Brown et al., 1994; Mandel and Yanofsky, 1995).

CArG-box motifs play a crucial role in initiating *FUL* transcription

The 3.9kb *FUL* regulatory region (-3938, -66) contains two CArG-box motifs – one located at -2512 (5' CArG) and one at -2038 (3' CArG) (see Fig. 5). Recall that when only one of the two CArG-boxes is mutated in the single mutation reporter constructs, *CArG-/+::GUS* and *CArG+/-::GUS*, the resulting transgenic lines are still capable of driving reporter expression (Fig. 7C, D). Though their expression levels and patterns differed slightly from those in wild-type controls, the loss-of-function of a single CArG motif from the *FUL* promoter did not greatly inhibit *FUL* transcription. However, when both CArG motifs were mutated in the *CArG-/-::GUS* constructs, transgenic lines displayed a striking phenotype where virtually no reporter signal could be detected throughout the plant (Fig. 7E). The only difference between the *FUL* promoter fragment used to drive *GUS* in *CArG-/-::GUS* constructs, and the wild type 3.9kb *FUL* promoter used to drive *GUS* in the *FUL::GUS* controls, is the mutation (and subsequent loss-of-function) of both CArG-box motifs. It thus appears that at least one functional CArG-box must be present within *FUL*'s regulatory region for any kind of *FUL* transcription to be initiated.

The specific MADS-box transcription factors that bind these CArG-box motifs to regulate *FUL* transcription is still unclear. Some MADS-box genes that may be involved with such regulation are the *AP1* and *AG* organ identity genes discussed above. Both have been shown to influence *FUL* activity, and both encode MADS-box transcription

factors capable of binding either CArG-box motif in the *FUL* promoter. Additionally, because the 5' and 3' CArG-boxes are less than 500 bp apart, and MADS factors can dimerize with one another, it is possible that the two CArG motifs work together to form a single tetrameric regulatory complex that is capable of driving *FUL* transcription.

More research is needed to determine exactly which MADS-box transcription factors bind either of the CArG-box motifs in *FUL*'s promoter. ChIP assays are useful in highlighting specific protein-DNA interactions, and may be used to test whether AP1 and AG, along with any other candidate MADS factors, directly bind *FUL*'s CArG-box motifs. Additional proteins likely bind these MADS factors, with the resulting tetrameric complexes providing tighter regulation of *FUL* transcription. Further studies that uncover such dimerizing factors are necessary in order to piece together the multifaceted roles the CArG-boxes play in regulating *FUL* activity.

Part II. Regulatory Roles of Auxin Response Elements in *FUL* Transcription

Auxin acts as a morphogen patterning the gynoecium

Auxin is an important phytohormone, playing a variety of regulatory roles throughout plant growth. Auxin naturally occurs in higher plants in the form of indole-3-acetic acid (IAA), which is synthesized in apical tissues of the plant and transported basally. This process requires directed auxin flow, which is mediated by the polar localization of auxin efflux carriers. Thus, an apical-basal gradient of auxin is generated by this polar auxin transport across the gynoecium primordium early in development (Chandler, 2010).

When polar auxin transport is disrupted, there is an accumulation of auxin near source cells and depletion in cells downstream of transport, thus increasing the slope of existing auxin gradients. In such cases, the pooling of auxin in apical primordium cells promotes precocious and excessive proliferation of style and stigma tissues, while the depletion of auxin in more basal primordium cells results in the loss of valve and ovules and lengthening of the stipe in the mature gynoecium. WT gynoecia treated with the polar auxin transport inhibitor, N-1-naphthylphthalamic acid (NPA), showed an increase in apical tissue proliferation (i.e. stigmatic and stylar tissue), a basalization of the style/ovary boundary, decreased valve production, elongated internode tissue, and a decrease in ovule development (Nemhauser et al., 2000).

In this sense, auxin acts a morphogen patterning the gynoecium, setting two boundaries, and therefore establishing three different domains, in gynoecium primordia. Threshold levels in the apical end of gynoecia mark the boundary between presumptive style and ovary tissue formation, with high levels of auxin specifying style and stigma tissue types. The low auxin levels in basal regions of gynoecia mark the boundary between presumptive ovary and internode tissues, with such minimal levels of auxin promoting gynophore and internode differentiation. The medial levels of auxin that exist between these two gynoecial boundaries pattern tissues of the ovary and thus promote valve development (Chandler, 2010; Nemhauser et al., 2000).

The Aux/IAA family of auxin-responsive genes

There are many different kinds of auxin-responsive genes whose activities are

rapidly induced by auxin, but all fall into three major classes – the *Aux/IAA*, *SAUR*, and *GH3* gene families (Hagen and Guilfoyle, 2002). There are 29 *Aux/IAA* genes in *Arabidopsis* that encode Aux/IAA proteins (Reed, 2001). Aux/IAA proteins share four conserved amino acid sequence motifs, referred to as domains I – IV. Domain I is the smallest and least conserved, with a proposed role in the homodimerization of Aux/IAA proteins. Domain II is highly conserved and functions to destabilize Aux/IAA proteins, lowering their overall activity. Domains III and IV form a dimerization platform, and mediate homo- and heterodimerization between Aux/IAA proteins and ARF proteins, discussed further below (Reed, 2001; Hagen and Guilfoyle, 2002). There is no evidence of Aux/IAA proteins binding specific DNA sequences – instead, these proteins dimerize with ARF proteins and thereby modulate auxin-regulated gene expression (Hagen and Guilfoyle, 2002).

Auxin Response Factors (ARFs)

Related to the *Aux/IAA* genes is a class of 23 *Arabidopsis ARF* genes, which encode auxin response factors (Hagen and Guilfoyle, 2002). While *ARF*s are not induced by auxin as in the *Aux/IAA* family, they are posttranscriptionally regulated by microRNAs (miRNA) and *trans*-acting-small interfering RNAs (tasiRNA) (Guilfoyle and Hagen, 2007).

ARF genes encode auxin response factors (ARFs), which function as transcription factors along with Aux/IAA proteins to regulate the expression of auxin response genes. Most ARFs consist of three conserved domains – an amino-terminal domain that serves

as a DNA-binding domain, a middle region that functions as either transcriptional activation or repression domains, and a carboxy-terminal domain that serves as a dimerization platform (Reed, 2001). The N-terminal DNA-binding domain specifically binds TGTCTC sequences, or auxin response elements (AuxREs), in the promoter regions of auxin response genes. AuxRE motifs are involved in the recruitment of transcription factors to certain promoters, and in the absence of auxin, AuxREs act to repress adjacent constitutive elements. The distribution of AuxREs throughout certain promoters may regulate the amplitude of the auxin response at those loci (Chapman and Estelle, 2009).

However, ARF proteins are targeted to AuxRE motifs in an auxin-independent manner, and do not regulate target gene activity in response to auxin by themselves. The ARF C-terminal domain is homologous to domains III and IV of Aux/IAA proteins, enabling the heterodimerization between ARF and Aux/IAA proteins. An auxin response requires a functional ARF C-terminal dimerization domain and the association of Aux/IAA protein with an ARF transcription factor (Guilfoyle and Hagen, 2007). Thus, the activity of ARF transcription factors is enabled through the auxin-dependent degradation of Aux/IAA repressors. In the absence of auxin, Aux/IAA proteins and corepressors are present at sufficient concentrations to repress activating ARFs (Chapman and Estelle, 2009).

The ARF activation and repression domains contain biased amino acid sequences. ARF activation domains are enriched in glutamine along with serine and leucine residues, while repression domains are enriched in serine, proline, leucine and glycine residues

(Guilfoyle and Hagen, 2007). When repressive ARFs are highly expressed, they displace endogenous ARFs on auxin-responsive promoters but direct a reduced level of promoter activation (Chapman and Estelle, 2009).

Regulation of auxin response gene expression must be highly complex because of the large family of ARF proteins that might compete for AuxRE target sites, as well as the potential interactions of ARFs with themselves and with the large family of Aux/IAA repressors. In general, when auxin concentrations are low, auxin response genes are repressed, likely as a result from the dimerization of Aux/IAA repressors with ARF transcriptional activators. When auxin concentrations are elevated, transcription is rapidly de-repressed, or activated, which is likely due to the dissociation of Aux/IAA repressors from their ARF counterparts and subsequent degradation of the Aux/IAA proteins by the ubiquitin/proteasome pathway. Recall that domain II of Aux/IAA repressors is involved in targeting the repressors for degradation in an auxin-dependent manner (Guilfoyle and Hagen, 2007; Hagen and Guilfoyle, 2002).

ETTIN (ETT), an Auxin Response Factor

ETTIN (*ETT*), also known as *ARF3,* is an auxin response gene that encodes proteins homologous to the previously discussed transcription factors capable of binding AuxRE motifs within promoter regions of auxin-responsive genes. However, unlike most ARF proteins, ETT transcription factors do not contain a carboxy-terminal dimerization domain related to domains III and IV of Aux/IAA proteins. Though ETT probably does not interact with Aux/IAA proteins, it may indirectly interact with such

proteins through competition among other ARFs for AuxRE binding sites. Despite this, we will focus on ETT as a possible regulator of *FUL* because of its early expression in the gynoecium and implicated roles in apical-basal patterning of abaxial gynoecial tissues (Reed, 2001; Sessions et al., 1997).

Dynamic *ETT* expression patterns

ETT expression is very dynamic and highly specified in particular floral tissues throughout flower development. *ETT* RNA is first detected in the inflorescence meristem at stage 1 of flower development, and resolves to the apex of floral meristems during stage 2. Within floral primordia, *ETT* is expressed throughout the inner three whorls of floral organs. It is expressed in petal primordia from stages 4-6, being restricted to procambial cells during stages 7-8, and ceasing by stage 9. *ETT* is expressed abaxially throughout stamen primordia from inception to stage 7, being reduced to vascular cells in stages 7-9 before diminishing by stage 9. Similarly, it is expressed in abaxial gynoecial tissues from inception until stage 8, and is refined to the four vascular strands of the gynoecium primordium during stage 9. All *ETT* expression is eliminated within gynoecia primordia, as well as from the rest of the flower, by stage 12 of flower development (Sessions et al., 1997).

***ett* mutant phenotypes**

When *ETT* function is eliminated, *ett* mutants display a wide range of pleiotropic effects on flower development. The numbers of sepals and petals are increased, while

stamen and anther form are decreased. The phenotypes for e*tt* gynoecia are allele-strength dependent, showing various alterations in the overall differentiation of gynoecial tissues. Three major defects can be observed in *ett* gynoecia: (i) a reduction in ovary size with an elongation of internode tissue, (ii) the appearance of stylar and stigmatic tissue types in the ovary region, and (iii) alterations in vascular patterning. The reduction in ovary size includes a significant loss of valve tissue, and is coupled with a basalization of stigmatic and style-like tissue in the apical region of the gynoecium, along with an elongation of internode (or stipe) tissue in the basal region. There is also a decrease in ovule production and female sterility may result. Additionally, *ett* gynoecia exhibit a trend towards basalization of vasculature patterns, displaying basalized expression of lateral bundle termination and medial bundle bifurcation (Sessions and Zambryski, 1995; Sessions et al., 1997; Nemhauser et al., 2000). These mutant phenotypes suggest that *ETT* is involved in apical-basal and abaxial-adaxial patterning of gynoecial tissues.

Proposed roles for *ETT* in flower development

The various floral defects observed in *ett* mutants implicate a dynamic role for *ETT* in patterning groups of cells within floral meristems. Early in floral development, *ETT* functions in determining the number of perianth organ primordia, following the increased number of sepals and petals observed in *ett* mutants (Sessions et al., 1997). Later in development, *ETT* is involved in patterning specific tissues within reproductive organ primordia, as seen by the defects in anther and carpel form in *ett* mutants (Sessions et al., 1997).

Abaxial *ETT* expression in the walls of the gynoecium primordium from inception until stage 8 likely performs three essential functions: (i) to promote formation of valve and ovary cell types, (ii) to repress formation of stylar and internode cell types to the apical and basal poles, and (iii) to pattern the sites of vascular differentiation (Sessions et al., 1997). Proper differentiation of tissues within the developing gynoecium occurs from two ringed boundaries established early in development, which is disrupted in *ett* mutants, as seen by a lowering of the apical (i.e., stigma and style forming) boundary and a raising of the basal (i.e., internode forming) boundary of stage 5 gynoecium primordium. ETT is proposed to establish these two regional boundaries, defining the apical and basal ends of the ovary (and thus the valves) during early gynoecium development (Nemhauser et al., 2000).

ETT function is necessary for the interpretation of positional information along the longitudinal and radial axes of developing gynoecia relative to boundary positions. Since ETT proteins contain a potential AuxRE DNA-binding site, they may be coordinating this information for positional tissue development in response to local levels of auxin concentration. In this model, ETT likely mediates the mid-level auxin response specifying valve development, while restricting high-level auxin responses specifying apical gynoecial tissues (Nemhauser et al., 2000). Furthermore, the range of allele strengths and phenotypes observed in *ett-1*, *ett-2* and *ett-3* mutants suggests that *ETT* patterns the gynoecium primordium in a dose-dependent manner, rather than by a simple threshold effect mechanism (Sessions and Zambryski, 1995).

ETT is post-transcriptionally regulated by *ZIP* genes via *trans*-acting small interfering RNAs (tasiRNAs). *ETT*, as well as *ARF2* and *ARF4*, are regulated by *TAS3*

tasiRNA, which target and cleave transcripts (Guilfoyle and Hagen, 2007).

ETT vs. *FUL* – Overlapping expression patterns and redundant mutant phenotypes

The similarity between mutant *ett* and *ful* fruit phenotypes suggests that the two genes have related or redundant functions, and may work together or in parallel pathways during fruit development. In addition, the overlapping expression patterns of *FUL* and *ETT* from inception to stage 12 of flower development, along with ETT's potential for binding two AuxRE sites within *FUL*'s promoter region, further suggests that the two genes interact to ensure proper fruit development and the differentiation of tissues within the ovary.

During vegetative development, *ETT* and *FUL* are both expressed throughout the leaves, stems, inflorescence meristem and vasculature tissue. As the plant transitions into flower development, *ETT* and *FUL* transcripts cultivate the floral meristem. While *ETT* remains active throughout petal, stamen and carpel primordium in stage 3, *FUL* activity localizes to fourth whorl carpel primordia only. As the carpel primordium develops and gynoecial tissues begin differentiating, *FUL* transcripts concentrate in lateral valve tissue of the ovary, with some style and gynophore expression within the gynoecium. Similarly, *ETT* is expressed in abaxial (outer) epidermal tissues of the gynoecium primordium from stages 5-9, when its expression becomes localized to vascular bundles before being eliminated completely by stage 12. After fertilization during stage 12, *FUL* activity in the valves concentrates to the apical (style-proximal) and basal (gynophore-proximal) poles of the ovary, and continues to be patterned in such an "apical/basal" or

"bipolar" expression gradient until its activity is diminishes in stage 17. The overlapping activities of *ETT* and *FUL* early in the gynoecium primordia, and later in abaxial carpel tissue suggests the two genes may work together to promote fruit development.

ett mutants have pleiotropic effects on flower development, causing an increase in sepal and petal number while negatively affecting stamen form and the differentiation of gynoecial tissues. Interestingly, *ett* and *ful* gynoecia share very similar mutant phenotypes, the most obvious being a shortened ovary with an elongated style and gynophore (or internode). The loss of *ETT* and *FUL* function each disrupt ovary growth, ultimately reducing valve development and preventing fruit elongation. The striking resemblance between *ett* and *ful* mutant gynoecia further supports the possibility that *ETT* may interact with and/or regulate *FUL* to promote proper fruit development, especially in the differentiation of abaxial gynoecial tissues.

ETT **as a possible regulator of** ***FUL*** **activity**

Since ETT transcription factors are capable of binding AuxRE motifs, in addition to the strong correlation between *ETT* and *FUL* expression patterns and *ett* and *ful* mutant phenotypes, ETT may bind either or both AuxRE motifs within the *FUL* promoter to regulate *FUL* transcription. As described earlier, *ETT* is involved with establishing the apical and basal boundaries of the ovary within developing gynoecia primordia, with implications in mediating the mid-level auxin response responsible for specifying valve tissue development. Thus, it is possible that *ETT* interacts with *FUL* to direct its activity to valve domains, promoting valve cell differentiation and development.

While ETT is a member of the ARF family of transcription factors, it does not contain a functional C-terminal domain that is responsible for dimerizing with Aux/IAA proteins to confer an auxin response. In the absence of auxin, Aux/IAA repressors are present in sufficient concentrations to repress ARF activators bound to AuxRE motifs within certain promoters, which in turn, represses adjacent constitutive elements and inhibits target gene expression (Chapman and Estelle, 2009). Although ETT proteins cannot interact with Aux/IAA repressors, they may mediate an auxin response in other ways. One possibility is that ETT interacts with other auxin-responsive transcription factors besides Aux/IAA proteins. For instance, SEUSS (SEU) proteins have been found to physically interact with ETT to regulate the transcription of auxin response genes involved in floral organ patterning (Pfluger and Zambryski, 2004; Bao et al., 2010). SEU may act as a bridging factor and its dimerization with an AuxRE-bound ETT transcription factor may be effective in conferring an auxin response to *FUL* transcriptional regulation, thereby limiting *FUL* activity to the valves.

More research is needed to test whether *ETT* and *FUL* physically interact, and if so, what kind of relationship they have. Reporter constructs provide a good system for visualizing expression patterns, and would be useful in preliminary dissection of *FUL* and *ETT* interactions. The *FUL* promoter can be used to drive *GUS* activity in *ett* mutant backgrounds, allowing one to visualize the *FUL* expression pattern that results from an absence of *ETT* regulation. If *ETT* functions an activator of *FUL* transcription, *FUL::GUS* signal in *ett* mutants should be lower than reporter levels in wild type controls since there is no ETT available to bind and activate *FUL* in *ett* mutants. Conversely, if *ETT* is involved with negative regulation of *FUL*, we should see reversed expression

patterns with higher *GUS* reporter signal in *ett* mutants relative to wild type backgrounds. The absence of ETT transcription factors in *ett* mutants would fail to repress *FUL* transcription, resulting in higher reporter levels and possibly expanded domains of *FUL* activity. In addition, ChIP assays would be useful in determining whether ETT transcription factors actually bind either or both AuxRE motifs in *FUL*'s promoter region.

It would also be interesting to see if/how each AuxRE within the *FUL* promoter functions independently in regulating transcription. Instead of using the full 3.9kb *FUL* promoter to drive *GUS* activity in *FUL::GUS* reporter constructs, various fragments of the promoter can be used that highlight the effects of different motifs on *FUL* activity. It is believed that the distribution of AuxREs along the regulatory regions of auxin response genes may affect the amplitude of the auxin response at those loci (Chapman and Estelle, 2009). Thus, it would be useful to create various *FUL* promoter reporter constructs that separate 5' and 3' AuxRE function, and compare *GUS* reporter levels in both WT and *ett* mutant backgrounds.

Part III. Motifs Involved in Regulating Valve-Specific Expression of *FUL*

"ABCD" Promoter Region

The "ABCD" *FUL* promoter region (-2971, -2512) was found to be particularly important for activating *FUL* transcription in the valves (Fig.'s 5 and 6). This 459 bp promoter fragment contains four candidate motifs (each representing a letter, "A" – "D") that may play critical roles in promoting *FUL* function in carpel valve development.

These valve-specific motifs are (from 3' to 5'): 5' CArG-box ("A"), MYB ("B"), SAUR ("C") and SBP ("D"). *ABCD::GUS* and *ABC::GUS* reporter lines display valve-specific expression patterns (Fig.'s 11 and 12), highlighting the smallest *FUL* promoter fragment yet found capable of driving *FUL* transcription in valve tissue.

The significance of CArG-box motifs in *FUL* regulation were previously discussed, with the presence of at least one functional CArG-box shown to be necessary for any *FUL* transcription to occur. Thus, the 5' CArG-box ("A") was included in all valve-specific reporter constructs. However, the specific roles that the MYB ("B"), SAUR ("C") and SBP ("D") promoter motifs play in controlling *FUL* activity in the valves remains unclear and are discussed in more detail below.

MYB ("B")

MYB genes encode a variety of transcription factors, categorized into three general groups based on the number of MYB repeats contained within the protein. Overall, MYB transcription factors are auxin-inducible and involved in many different developmental processes from cell division and differentiation to secondary metabolism and the biosynthesis of phenylpropanoids (Chapman and Estelle, 2009; Maeda et al., 2006; Stracke et al., 2001; Romero et al., 1998).

The 3.9kb *FUL* promoter region contains an MYB motif that is capable of binding MYB transcription factors. This MYB motif is located 2686bp upstream the *FUL* start codon, and represents the "*B*" in *ABC::GUS* and *ABCD::GUS* transgenic lines. Though no constructs have yet been made that isolate MYB's role in regulating *FUL*

transcription, we predict this motif is involved in controlling auxin-mediated valve cell differentiation, based on the typical roles MYB transcription factors play in flower development.

One MYB protein that may bind *FUL*'s promoter to regulate its activity in valve development is AS1. *ASYMMETRIC LEAVES1* (*AS1*) genes encode MYB transcription factors and are involved in the medio-lateral patterning of fruit (Girin et al., 2009). Within the gynoecium, *AS1* transcripts are detected at high levels in the valves and at low levels in the replum. In the valves, *AS1* is proposed to repress *BP* and related *KNOX I* genes involved in replum development, thus promoting valve formation. *as1* mutants display enlarged repla and reduced valve regions, underlining the role of *AS1* in promoting valve initiation. Furthermore, *as1 ful* double mutants displayed extremely small valves with very large, distorted repla, indicating a strong enhancement of the phenotypes of the two single mutants both in valves and replum (Alonso-Cantabrana et al., 2007; Girin et al., 2009). The synergistic interaction between *as1* and *ful* mutant alleles makes AS1 a good candidate for *FUL* regulation by binding to the MYB motif contained within the *FUL* promoter.

To see whether AS1 actually binds the *FUL* promoter to positively regulate transcription and promote valve development, ChIP assays could be performed with AS1 proteins and *FUL* promoter region DNA. If AS1 interacts with *FUL*, additional *FUL* promoter constructs should be created to analyze the role of the MYB motif in *FUL* transcriptional regulation. Such constructs may include *ACD::GUS* (similar to the *ABCD::GUS* constructs except for the missing MYB motif, aka "*B*") and *MYB*$^{-}$*::GUS* (in

which the 3.9kb *FUL* promoter contains a point mutation at the MYB motif that renders it unable to bind AS1 transcription factors).

SAUR ("C")

The first *SAUR* (*small auxin-up RNA*) genes were characterized in soybean, with at least 72 *SAUR* genes already identified in *Arabidopsis*. *SAUR* transcripts accumulate in cells that are destined to elongate, most likely from the redistribution of endogenous auxin (Gil et al., 1994; Park et al., 2007). Based on their expression properties, the *SAUR* genes have been implicated in auxin-induced cell elongation, and their transcripts are constitutively unstable so that their abundance can be rapidly altered in response to transcriptional control by auxin (Johnson et al., 2000). The instability of *SAUR* transcripts is due to sequences downstream the coding region, in the 3' untranslated region (UTR), of *SAUR* genes. This downstream element (DST) is approximately 40-45 bp long and consists of three highly conserved sequences separated by two variable regions. These DST sequences act to destabilize *SAUR* transcripts so as to allow *SAUR* mRNA levels to adjust rapidly in response to increases or decreases in auxin concentration (Gil et al., 1994; Newman et al., 1993). Because auxin plays a central role in many aspects of plant development, there may be a narrow tolerance for misexpression of such auxin response genes.

***SAUR* Regulation of *FUL* Activity in Valve Development**

Perhaps the best-characterized gene in *Arabidopsis* that is rapidly induced by

auxin is *SAUR-AC1*. Like most *SAUR*s, *SAUR-AC1* mRNA contains the conserved DST instability sequence in the 3' UTR, and *SAUR-AC1* transcript accumulation is readily induced by natural and synthetic auxins, as well as cycloheximide. *SAUR-AC1* promoter activity is present in aerial tissues of seedlings, with the most prominent activity seen in elongating hypocotyls, floral stems, vasculature cells and tissues throughout the flower (Gil et al., 1994; Gil and Green, 1997).

The *FUL* promoter contains a *SAUR* motif, capable of binding SAUR transcription factors, and is located 2893 bp upstream *FUL* ATG in the promoter region previously believed to be crucial for *FUL*'s expression in the valves (Nguyen, 2008). Within this "ABCD" valve-specific promoter fragment, the *SAUR* motif represents "C" and is present in the *ABC::GUS* and *ABCD::GUS* constructs (Fig.'s 5 and 6).

Since *SAUR-AC1* and *FUL* have overlapping expression domains in the valves of developing gynoecia, it is possible that SAUR-AC1 transcription factors bind the *SAUR* motif within *FUL*'s promoter region to influence *FUL* transcription in the valves. Though *FUL* promoter constructs isolating *SAUR* function have not yet been analyzed, it is likely that SAUR proteins bind this promoter region to influence *FUL* activity in response to levels of auxin, and may infer regional auxin sensitivity to valve cell elongation by *FUL*.

To confirm these hypotheses, *AB::GUS* (where only the 5' CArG-box, or "A", and MYB motif, or "B", from the *FUL* promoter are used to drive *GUS* expression) and *ABD::GUS* (5' CArG, MYB and SBP motifs used to drive *GUS*) transgenic lines shall be analyzed. The resulting *GUS* expression patterns from these "SAUR-deficient" lines can be compared with those from the previously evaluated "SAUR-containing" lines, such as

ABC::GUS, *ABCD::GUS* and *FUL::GUS* (WT) lines. Differences in gynoecium expression patterns among these transgenic lines may better emphasize the role of SAUR in *FUL* transcriptional regulation.

SBP ("D")

SQUAMOSA (*SQUA*) is a member of the MIKC group of MADS-box genes in *Arabidopsis* that specify flower meristem identity (Xie et al., 2006). *SQUAMOSA* promoter-binding-like (*SPL*) genes are plant-specific, with 16 different *SPL*'s identified in *Arabidopsis* so far. *SPL*'s encode *SQUAMOSA* promoter-binding proteins (SBP), all of which share a common stretch of 80 amino acids. This conserved region, referred to as the SBP domain, is believed to contain a novel zinc finger motif and serves as the DNA-binding domain (Xie et al., 2006). SBP transcription factors have been shown to bind with specificity to the promoter regions of target genes, with the SBP domain binding the consensus sequence, TNCGTACAA (where N = any base). Though the SBPs are a structurally heterogeneous family of transcription factors, they are all primarily involved with promoting floral development, especially in transitioning from (adult) vegetative phases to reproductive (or floral) phases (Schwarz et al., 2008; Yamasaki et al., 2004).

SPL* Genes in *Arabidopsis

Currently, not much is known about each of the *SPL*'s in *Arabidopsis*, but recent studies have begun to unveil the functions of individual *SPL*'s and strongly suggest a role in regulating several floral identity genes. Thus, it is highly likely that *SPL* and *FUL* work together to promote proper flower formation.

SPL9 is upregulated during the vegetative phase change, and promotes the transition from juvenile to adult vegetative phases. It does this by activating certain microRNA's involved in post-transcriptional gene silencing at different stages of development. *miR156* represses a number of *SPL* genes, and causes a delay in flowering. *SPL9* activates *miR156* precursors during juvenile phases, thus prolonging vegetative development. Later on, *SPL9* ceases *miR156* activation, and binds to the promoters of *miR172* precursors to promote *miR172* transcription (Schwarz et al., 2008; Fornara and Coupland, 2009). *miR172* has complementary functions and (temporal) expression patterns as *miR156*, and promotes juvenile-to-adult phase transitions. Thus, *SPL9* forms a negative feedback loop regulating the levels of *miR156* and *miR172*, and controlling the timing of the vegetative phase change (Fornara and Coupland, 2009).

SPL3 is expressed during vegetative development and is strongly upregulated in the transition from vegetative to reproductive development. Its expression is post-transcriptionally regulated by *miR156*, repressing *SPL3* early in vegetative development (Cardon et al., 1999; Unte et al., 2003). Overexpression of *SPL3* induces early flowering in transgenic plants, causing them to develop fewer rosette leaves and secondary inflorescences. In addition, *SPL3* activity is responsive to the photoperiod pathway, with transcript levels increasing when plants are moved from short to long day growth conditions. Levels of *SPL3* increase when the photoperiod regulator, *FT*, is elevated. These two genes work in parallel pathways to upregulate meristem identity (MI) genes and induce flower formation. *SPL3* has been shown to bind the promoter regions of MI genes, such as *LFY*, *FUL*, and *AP1*, to control the onset of floral development (Fornara and Coupland, 2009; Yamaguchi et al., 2009).

SPL Regulation

In general, the SBP family of transcription factors activate floral meristem identity genes to promote reproductive development and the formation of flowers. As a result, *SPL* repression by *miR156* is crucial for maintaining vegetative development. Levels of *miR156* are heavily dependent on the genes involved in post-transcriptional gene silencing (PTGS), such as *ZIP* (member of *AGO* family), *RDR6* (necessary for miRNA transcription), and *DCL4* (required to process functional miRNAs) (Fornara and Coupland, 2009; Schwarz et al., 2008). Hence, endogenous regulation of *SPL* (and thus flowering) rests on PTGS genes synthesizing *miR156*, which represses *SPL*s and delays the transition to reproductive development. Loss-of-function mutations in PTGS genes, such as *ZIP*, result in upregulation of SBP transcripts and precocious flowering (Yamaguchi et al., 2009).

The photoperiod pathway is sensitive to day length and provides a means for environmental regulation of flowering. Regulatory genes involved in this pathway include *FT* and *FD*, which activate floral meristem identity genes under long day conditions and promote flower development. While photoperiod regulators promote flowering by activating *SPL*s and MI genes (e.g. *FUL*), *SPL* transcripts are also capable of indirectly activating photoperiod regulators via upregulation of *miR172* (Yamaguchi et al., 2009; Fornara and Coupland, 2009). This not only creates a self-sustaining pathway of reproductive development, but it also provides a way for *SPL* transcripts to overcome *miR156* repression.

SBP Transcription Factors and *FUL*

Given that one of *SPL*'s functions is to activate floral meristem identity genes, it is highly likely that the SBP motif contained within the *FUL* promoter is a site for *SPL* transcripts to bind and stimulate *FUL* transcription during the transition to flower development. This hypothesis correlates with the results seen from the "ABCD" constructs (Figs 11,12). The *ABCD::GUS* transgenic lines (*GUS* constructs driven by *FUL* promoter fragment including the SBP motif) showed higher levels of *FUL* transcripts in carpels than WT plants, with ectopic *FUL* expression in valve tissue of young fruits. In contrast, *ABC::GUS* transgenics (*GUS* constructs driven by *FUL* promoter fragment lacking the SBP motif) had very faint to no *FUL* expression in the valves (Fig. 11). The lack of *FUL* expression in *ABC::GUS* mutant fruit is most likely due to the lack of the SBP motif in the *FUL* promoter, preventing SBP transcripts from binding and activating *FUL* transcription. However, the faint *FUL* expression detected in *ABC::GUS* fruit shows that although *SPL* is sufficient in activating *FUL* transcription, it is not absolutely necessary and there exists at least one other transcription factor responsible for driving *FUL* transcription in the valves. This additional activator must bind a motif within the *FUL* promoter fragment used to create the *ABC::GUS* constructs. Good candidates for this would be one (or more) of the three motifs symbolized by "A," "B," and "C" – namely the (5') CArG box, MYB, or SAUR motifs (Fig. 5). On the other hand, the upregulation of *FUL* in *ABCD::GUS* gynoecia suggests that there are repressors of *FUL* that bind motifs outside the coordinates of the "ABCD" promoter fragment. Thus, these *FUL* repressors are rendered nonfunctional and fail to down-regulate *FUL* transcription in *ABCD::GUS* constructs, causing *FUL* expression levels to be elevated.

SBP and ARF Transcription Factors in *FUL* Regulation

Recall the two AuxRE motifs positioned at either end of the *FUL* promoter which, hypothetically, bind ARF transcription factors. As described earlier, the proposed function of these ARF transcripts (such as ETT) is to guide/pattern *FUL* expression in the valves (i.e. apical/basal gradient) by repressing *FUL* transcription in particular locations during early floral development. It should be noted that *FUL* expression in the valves of *AuxRE* (especially *AuxRE+/-*) and *ABCD::GUS* transgenic lines all resulted in a general increase in transcript levels (higher than WT) with a loss of the apical/basal patterning along the longitudinal fruit axis (Figs. 9-12). This is not surprising, as both sets of constructs lack functional AuxRE motifs (whether mutated in *AuxRE::GUS* or missing in *ABCD::GUS*) but still retain the SBP motif (Fig. 6). Thus, the increased valve expression is partly due to SBP activation of *FUL* at the onset of flower development, in addition to the absence of repression by ARFs (e.g. ETT), who are unable to bind either AuxRE motif. The inability of ARFs to bind *FUL*'s promoter is also the cause of the ectopic valve expression seen in *AuxRE::GUS* and *ABCD::GUS* transgenic fruit. Though *FUL* activity is high, it does not retain the polar expression gradient seen in WT valves, since the ARFs are incapable of setting boundaries for organ dimensions and directing *FUL* in valve development.

These principles also correlate with *FUL* activity seen in *ABC::GUS* fruit. These transgenic gynoecia, lacking the SBP motif in addition to both AuxRE motifs, showed a significant reduction in valve signal. The drastic decline in transcript *levels* is partly caused by the failure of SBP to upregulate activity at the onset of flowering. However, the fact that some signal can still be detected in the valves suggests that,

though SBP is an important activator of *FUL* transcription, it is not solely responsible for all *FUL* expression. The low valve signal may either be due to activator(s) binding the "ABC" promoter region and driving transcription, or a lack of repressors capable of binding *FUL*'s promoter to inhibit transcription. . Good candidates for additional activators include the 5' CArG-box (*A*), MYB (*B*), and/or SAUR (*C*). The lack of repressors may be due to the missing AuxRE motifs, which are involved in ARF repression. The loss of transcript *patterning* in *ABC::GUS* valve tissue is due to the lack of ARFs setting limits for regional gene activity, which likely contributes to the apical/basal expression gradient in the valves of WT *FUL*.

APPENDIX A: FIGURES

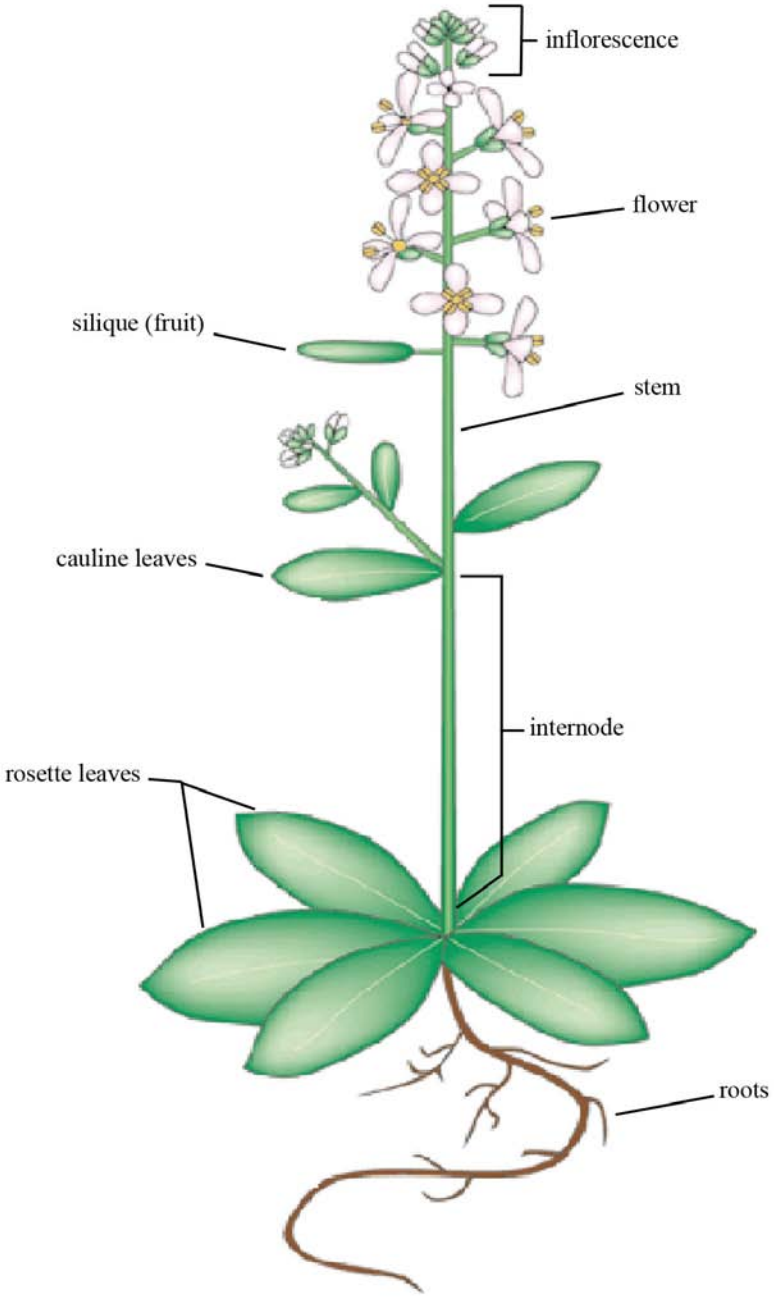

Figure 1. Basic Anatomy of *Arabidopsis thaliana*
The whole plant consists of a central shoot (or stem), from which smaller lateral shoots branch out. Each shoot contains an inflorescence that produces several flowers. After fertilization, seeds begin developing within the carpels of flowers, and this structure becomes the silique, or mature fruit.

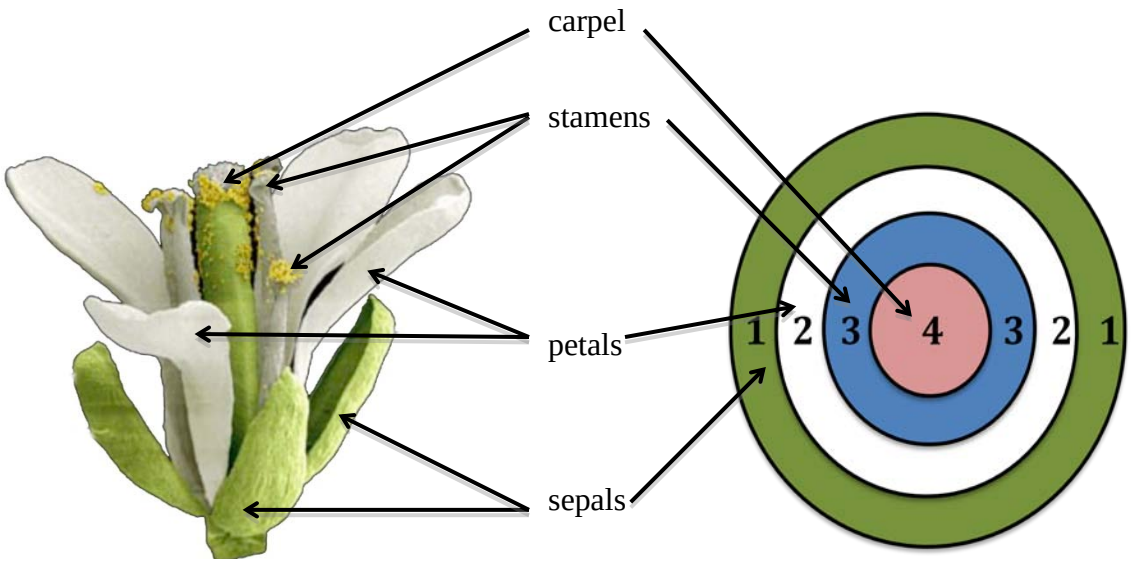

Figure 2. Flower Anatomy and the Four Whorls of Organs.
Each flower consists of four different organs arranged in concentric rings, or whorls, around its central axis. The diagram on the left shows how each organ appears on an actual flower, while the schematic on the right depicts how each organ whorl is positioned within the flower (as viewed from above). Note how the male and female reproductive organs are located within the two innermost whorls – six stamens in Whorl 3 (male) and two fused carpels in Whorl 4 (female).

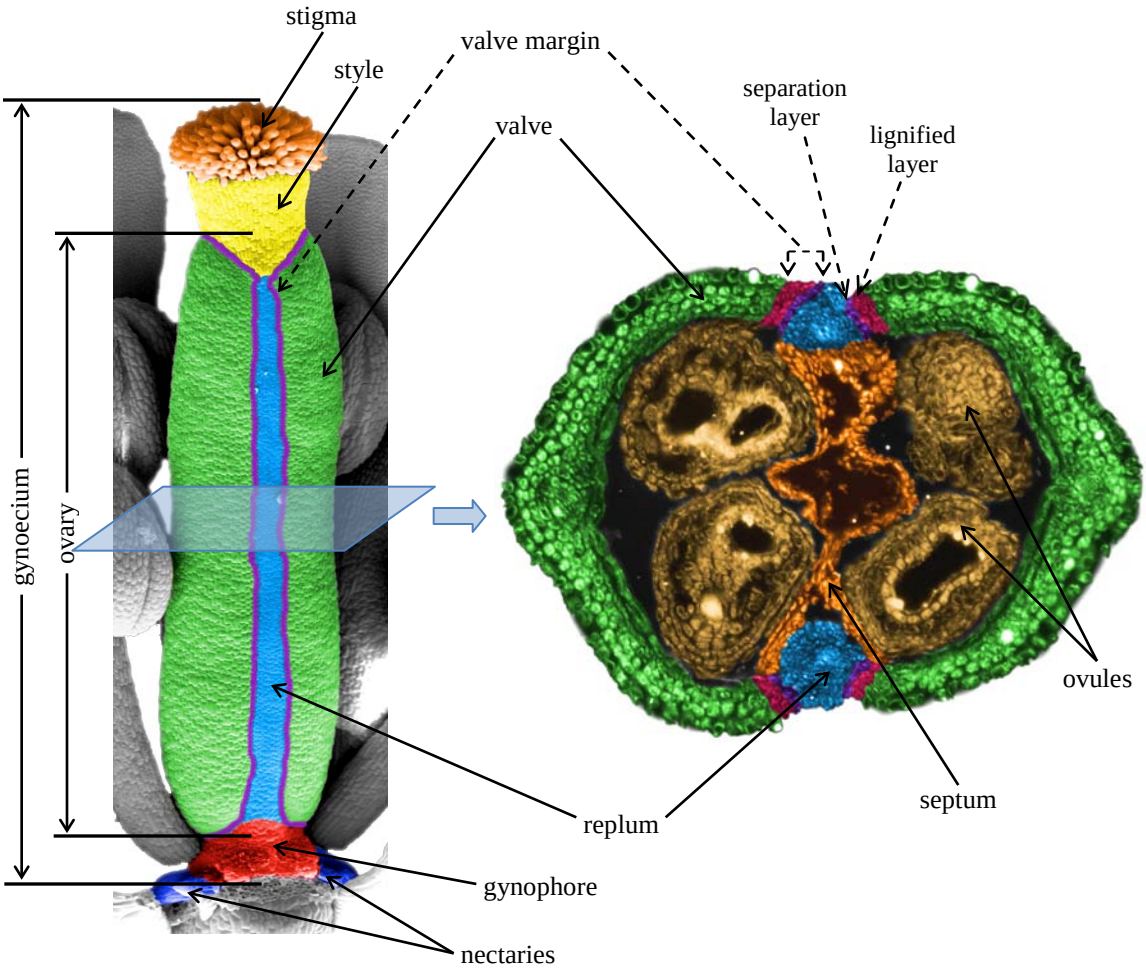

Figure 3. Gynoecium Structure and Tissues of the Ovary
The figure on the left is a scanning electron micrograph (SEM) of an *Arabidopsis thaliana* gynoecium (or carpel), with each color representing a different cell type. The figure on the right is a transverse cross section of a stage 13 ovary (post-fertilization). Two lateral valves (green) and medial replum tissue (blue) make up the outer walls of the ovary. Valves and repla are connected by a thin strip of valve margin tissue (made up of two distinct cell types, purple and pink), which is crucial for dehiscence and the release of mature seeds.

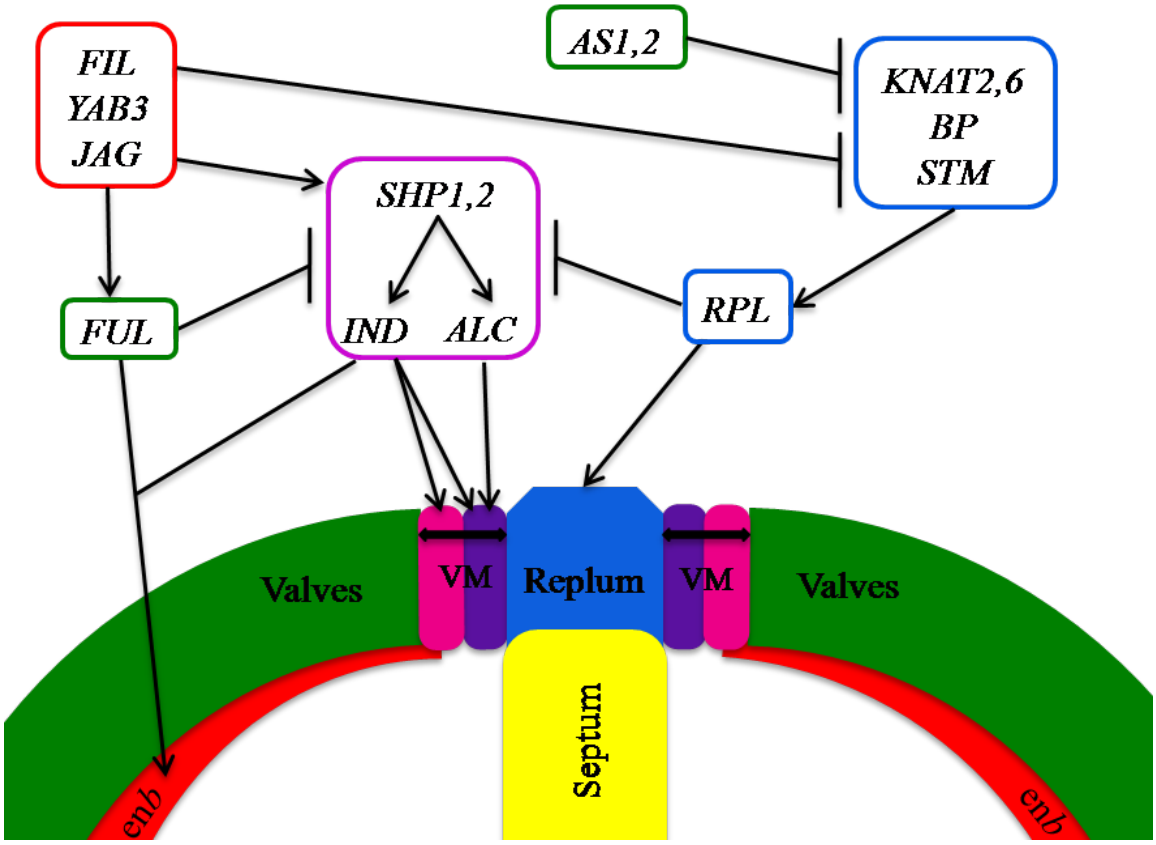

Figure 4. Genetic Network Patterning Fruit Development.
Many genes interact to pattern the tissues of the gynoecium. *FUL* regulates valve growth by repressing valve margin genes (*SHP1,2*, *IND*, and *ALC*) from being expressed in the valves, while *RPL* regulates replum growth by repressing those genes in the replum. The combined activities of valve (*FUL*) and valve margin genes creates the lignified en*b* layer of valves. Several upstream genes, such as *FIL*, *YAB3*, *JAG* and the class I KNOX genes (*KNAT2,6*, *BP*, and *STM*), regulate and pattern the activities of these primary genes.

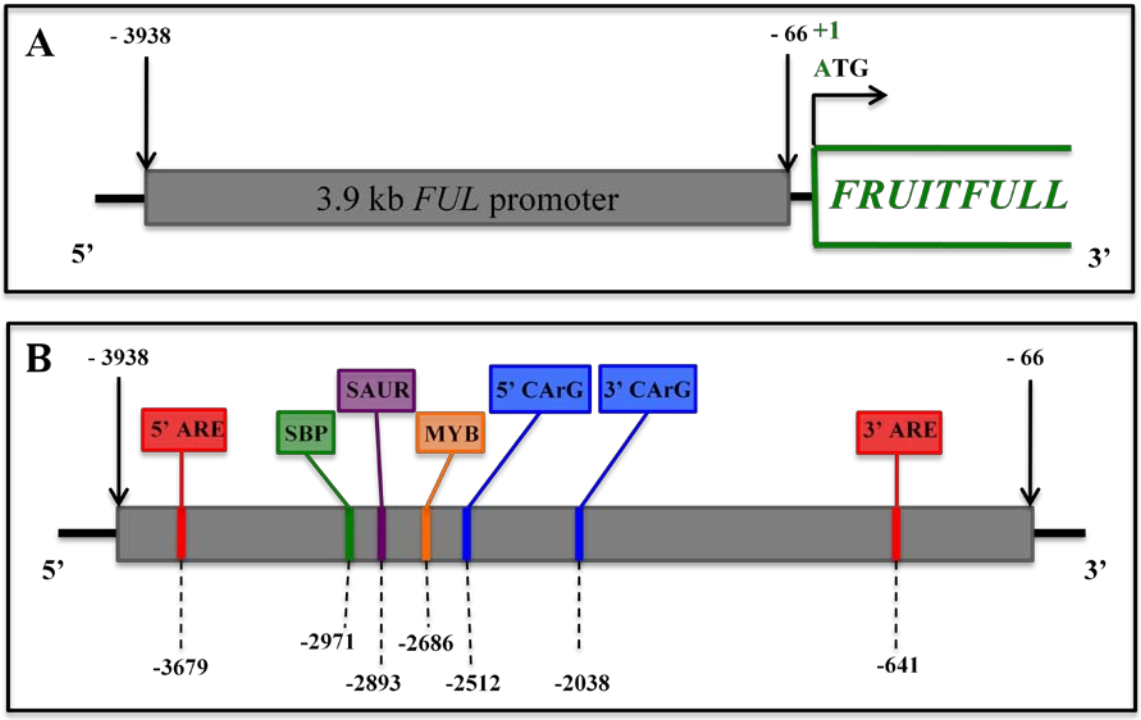

Figure 5. The 3.9 kb *FUL* Promoter and its Motifs.
A) The coding region of *FUL* (AT5G60910) is indicated in green and its 3.9 kb upstream regulatory region (or promoter) is in gray. Coordinates are given as the number of base pairs from *FUL*'s start codon – downstream (3') positions are positive (adenine, or A, of ATG = +1); upstream (5') positions are negative.
B) The positions of seven different promoter elements (or motifs) are shown within the 3.9 kb promoter region – these are putative regulators of *FUL* transcription. Four of these motifs, denoted "ABCD" for short, are believed to be specifically involved with regulating *FUL* activity in valve tissue. The "ABCD" motifs are: 5' CArG-box ("A"), MYB ("B"), SAUR ("C"), and SBP ("D").

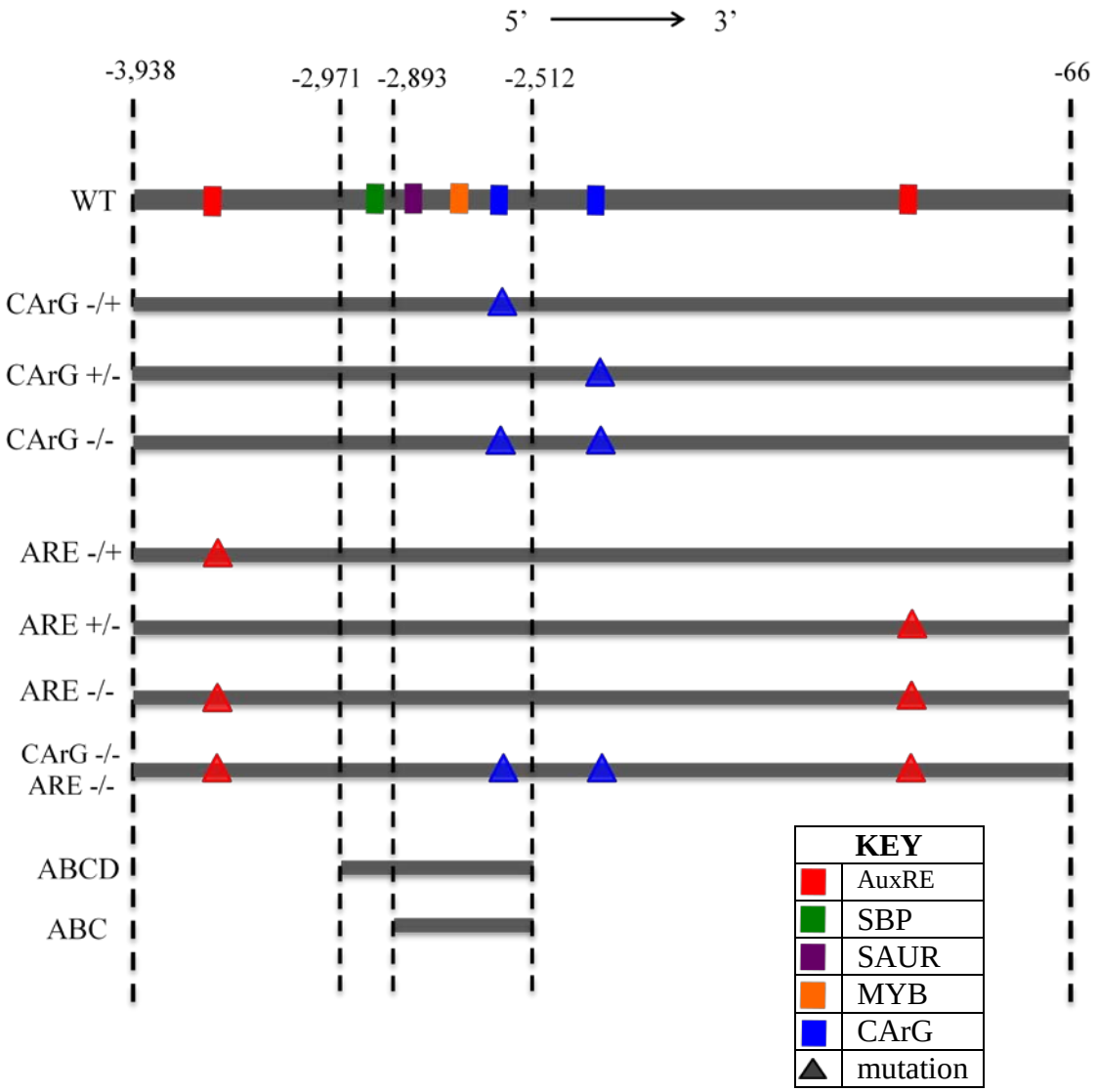

Figure 6. *GUS* Promoter Constructs.
Various fragments of the *FUL* promoter were isolated and cloned into pDW294 vectors to drive *GUS* expression. These promoter fragments are shown above, with their corresponding reporter line names to the left. Coordinates are given as the number of base pairs away from *FUL* ATG (negative values denote positions upstream the *FUL* coding region). The wild type (WT) 3.9 kb *FUL* promoter is on top, highlighting the seven motifs of interest. The promoter fragments below portray alterations made to the WT promoter, with triangles representing point mutations. *ABCD::GUS* and *ABC::GUS* constructs employ truncated promoter fragments, having only 459 bp and 381 bp driving *GUS* activity, respectively.

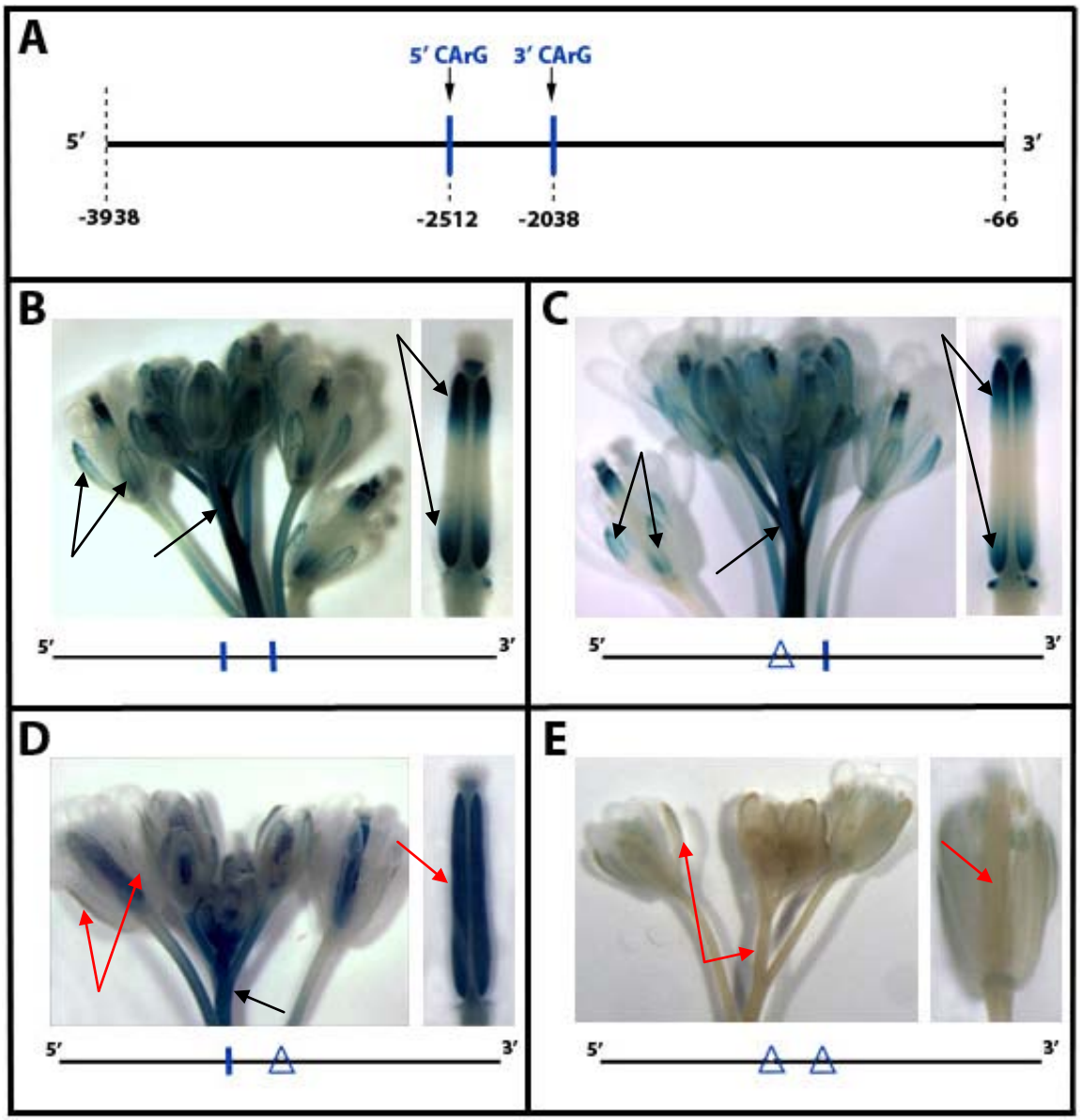

Figure 7. *CArG::GUS* Reporter Lines.
(A) WT 3.9 kb *FUL* promoter (-3938, -66) with both 5' and 3' CArG-boxes highlighted in blue. (B-E) Whole mount pictures of inflorescence and stage 13 fruit. (B) *FUL::GUS* (*CArG* +/+) lines serve as WT *FUL* control – note expression in inflorescence stem, sepal vasculature, and bipolar gradient in valve tissue. (C) *CArG -/+::GUS* lines, harboring a 5' CArG mutation, are able to maintain WT-like expression in the inflorescence and fruit. (D) *CArG +/-::GUS* flowers (having a 3' CArG mutation) show a loss of expression in sepal vasculature, and fruits show uniform *GUS* signal throughout the lengths of valves (no bipolar gradient). (E) *CArG -/-::GUS* lines show almost no *GUS* activity anywhere, except for faint signal detected in anthers and stigma.

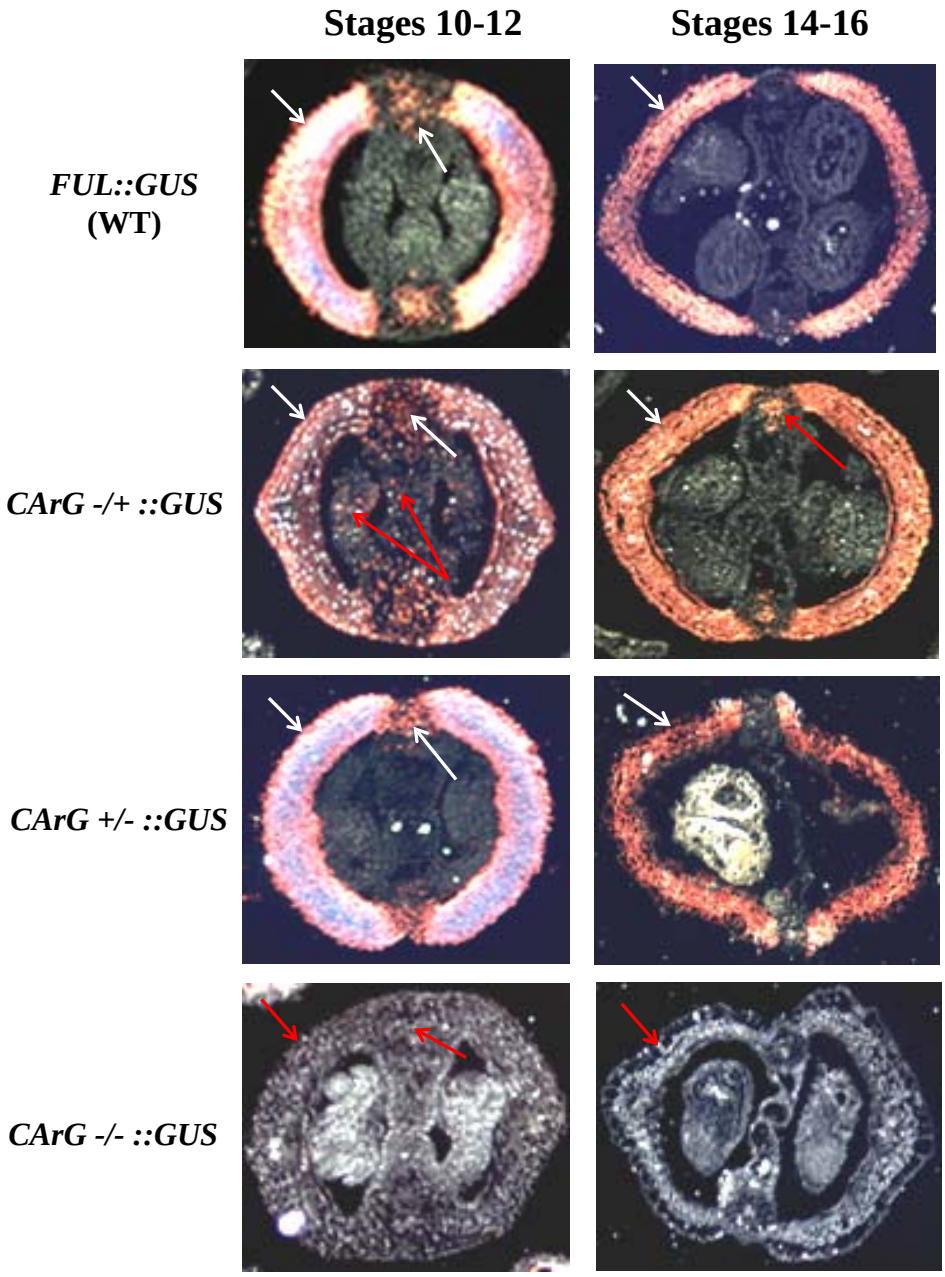

Figure 8. *CArG::GUS* Reporter Lines.
Analysis of expression patterns seen in cross sections of stages 10-12 (pre-anthesis) and stages 14-16 (post-anthesis) gynoecia. *FUL::GUS* lines portray WT *FUL* expression patterns – note how *FUL* activity is primarily valve-specific, with some medial replum activity detected in pre-anthesis pistils. Young *CArG -/+::GUS* gynoecia (5' CArG mutation) display ectopic ovary expression and retain the medial replum signal after anthesis. *CArG +/-::GUS* lines (3' CArG mutation) appear WT-like. *CArG -/-::GUS* lines (double CArG mutation) fail to show any signal whatsoever within fruit tissues.

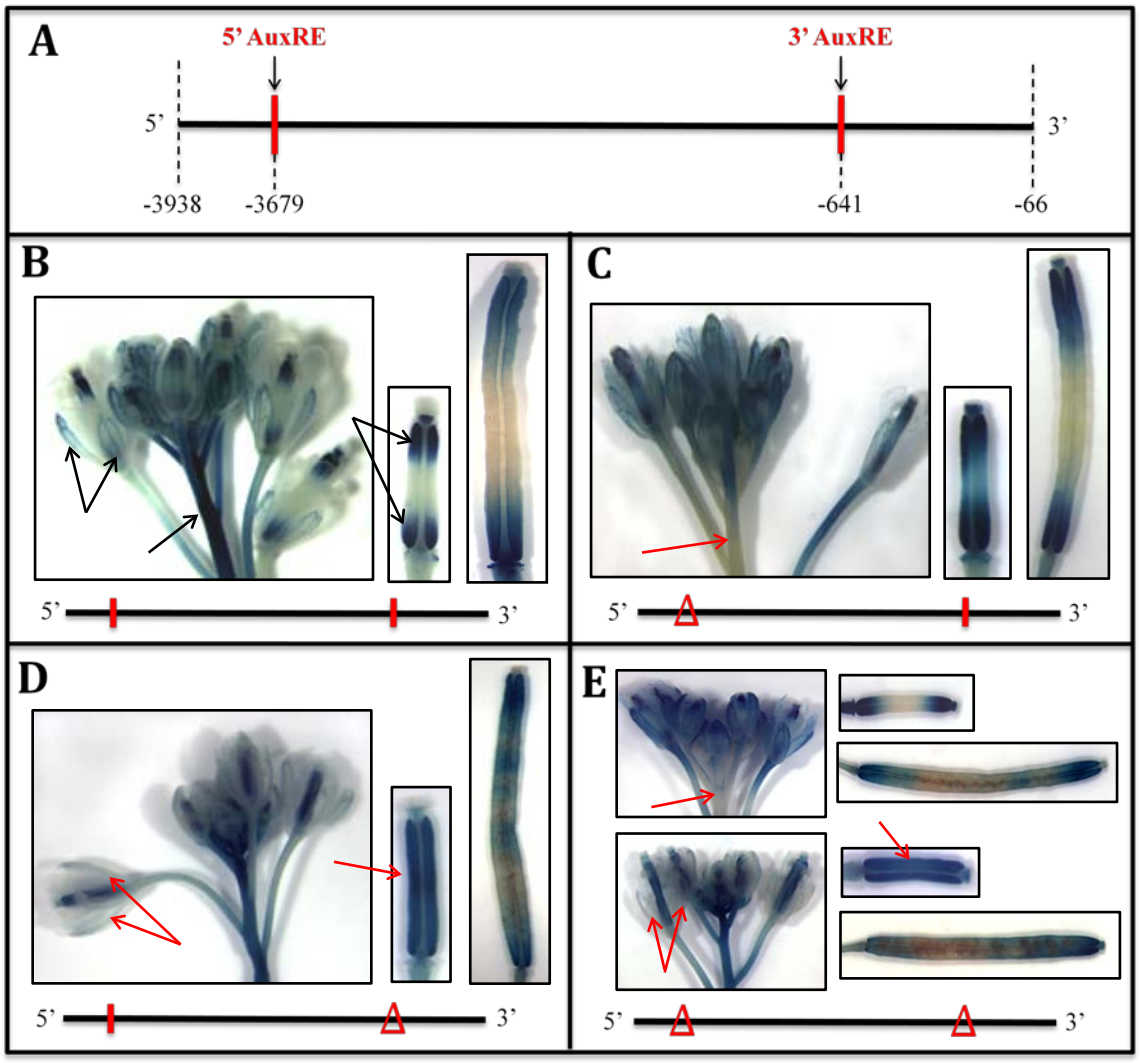

Figure 9. *AuxRE::GUS* Lines
(A) WT 3.9kb *FUL* promoter with positions of auxin response elements (AuxREs) highlighted in red. (B-E) Whole mount pictures of inflorescences, and stage 13 and 17 fruit. Below each figure is a diagram of the *FUL* promoter fragment used to drive *GUS* expression (Δ = mutated AuxRE). (B) *FUL::GUS* lines display WT-like expression patterns. (C) In *AuxRE -/+::GUS* lines (5' AuxRE mutation), signal is reduced in the stem, though fruit expression appears WT-like. (D) *AuxRE +/-::GUS* fruit (3' AuxRE mutation) lack the WT-like bipolar expression gradient in valve tissue. Also, sepal vasculature signal is reduced. (E) When both AuxREs are mutated in *AuxRE -/-::GUS* lines, no definitive expression pattern results. Some fruits display bipolar expression gradients (top), while others do not (bottom).

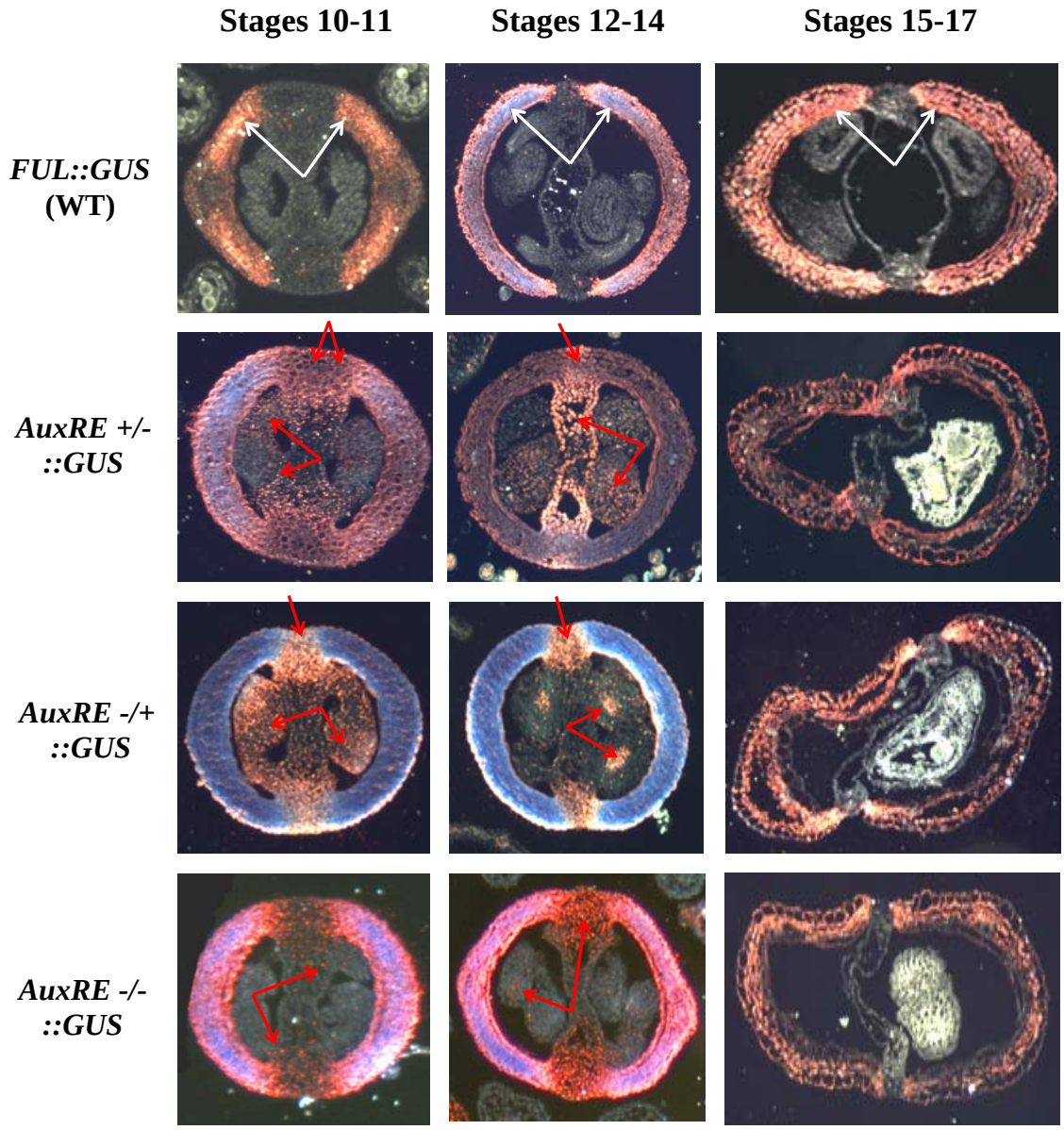

Figure 10. *AuxRE::GUS* Lines
Cross sections of gynoecia from various stages of development, indicated at the top of each column. Compare WT-like *FUL* expression (top) to those of the transgenic lines harboring one or more AuxRE mutation (below) – all AuxRE mutations (5', 3' or both) cause ectopic expression in ovule primordial (red arrows), especially noticeable in younger gynoecia (st. 10-11). Note: a red GUS stain represents lower signal intensity than a blue or purple stain. Thus, overall expression levels appear higher in AuxRE mutants compared to WT.

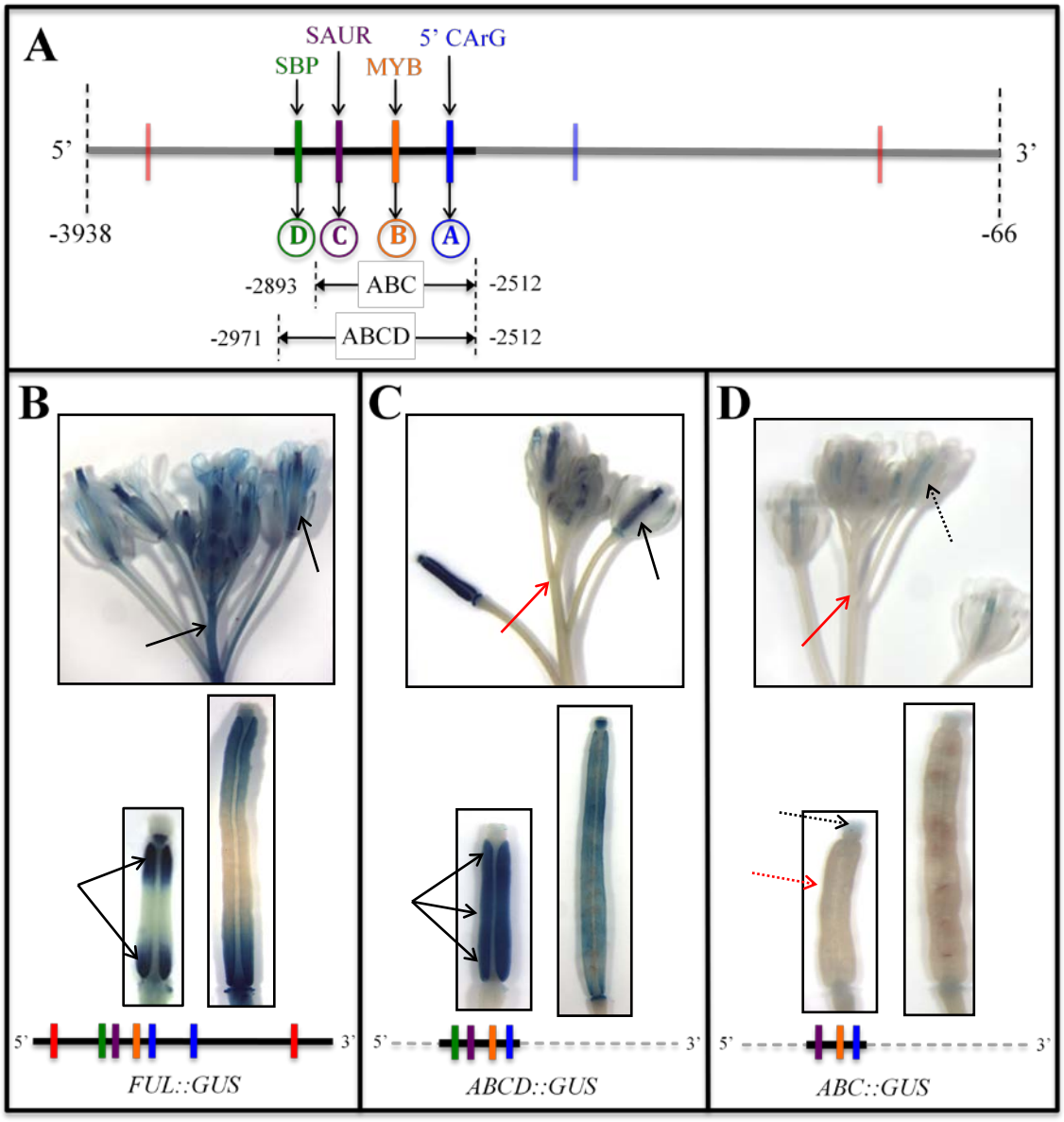

Figure 11. The SBP Motif and Valve-Specific Expression
(A) WT 3.9kb *FUL* promoter highlighting the region necessary for *FUL* transcription in the valves.[1] The only difference between the "ABC" and "ABCD" region is the SBP motif (or "D"). (B-D) Whole mount inflorescences (top), and stage 13 (left) and 17 (right) fruit. (B) WT *FUL* expression pattern is concentrated at the poles of fruit tissue. (C) *ABCD::GUS* fruit have strong valve-specific expression (no signal anywhere else), though the WT-like bipolar gradient is lacking. (D) *ABC::GUS* lines have severely reduced valve expression (barely visible through inflorescences), suggesting that the SBP motif is significant for maintaining *FUL* transcription in valve tissue.

[1] Nguyen, 2008.

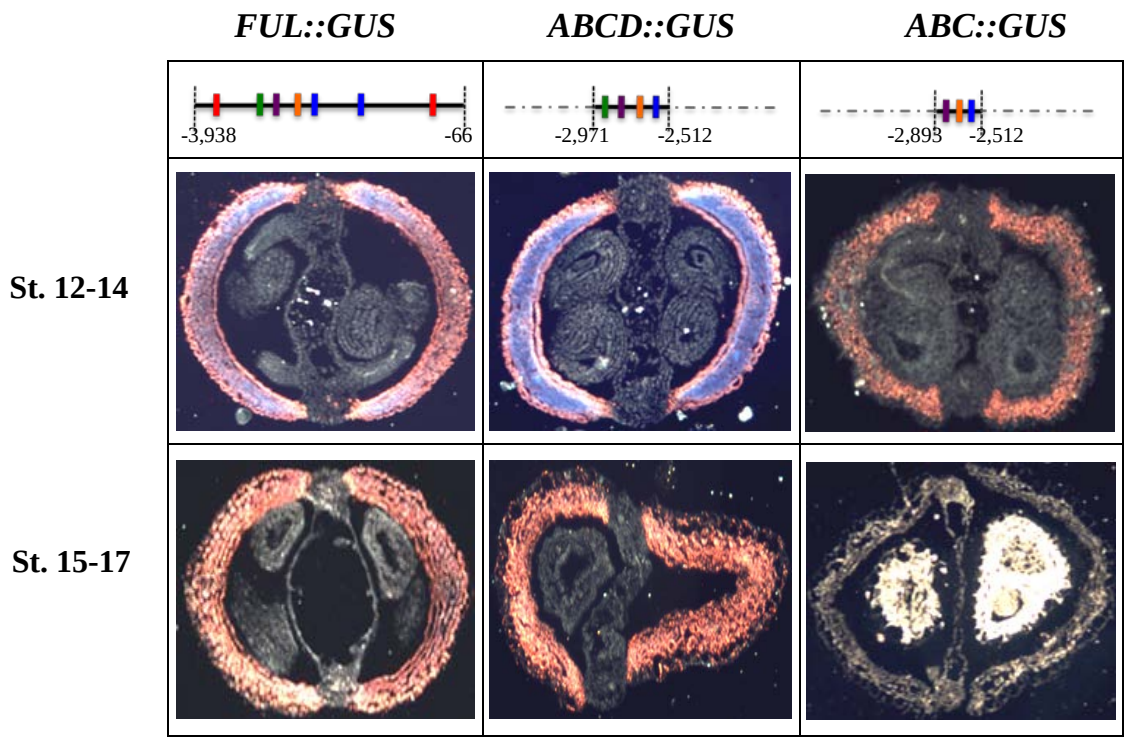

Figure 12. The SBP Motif and Valve-Specific Expression
Cross sections of gynoecia from various stages of development, indicated to the left of each row. Top row indicates the *FUL* promoter fragment (along with promoter coordinates) used to drive *GUS* expression in reporter constructs. Note how *ABCD::GUS* (middle) and *ABC::GUS* (right) fruit both display WT-like (left) expression patterns, though their signal intensities differ. Valve tissue in *ABCD::GUS* lines portray similar signal intensities as WT, while those in *ABC::GUS* lines have significantly reduced levels, especially noticeable in older fruit.

APPENDIX B: TABLES

Oligo	Sequence 5' → 3'	RE	Description
oJW1	TTGGATCCCACTCGTCCGACTA AAATGTATG	BamHI	3' border of "ABC(D)" constructs
oJW2	TTCTGCAGGGTCACACACAGAA AAAATAGAC	Pst	5' border of "ABC" constructs (used w/ oJW1)
oJW8	TTCTGCAGTCATGATGAAGGCC CTTATGGTG	PstI	5' border of "ABCD" constructs (used w/ oJW1)

Table 1. Oligonucleotides Used to Create "ABCD" *GUS* reporter constructs
The following oligonucleotides were used for the PCR-amplification of "ABC" and "ABCD" *FUL* promoter fragments from pAN1. Each promoter fragment produced contains a restriction enzyme site on either end to allow its insertion into the pDW reporter constructs. Restriction sites within each oligonucleotide sequence are underlined, with the corresponding restriction enzyme (RE) given in the next column.

Oligo Name	Oligo Sequence 5' → 3'	Motif Mutated	WT Motif Sequence (5'→3')
oAN29	CTTTTTGTTCATGTGGT**GG**<u>**AATTTT**</u>**CC**TATAT ACTATATAGTCTATAG	5' CArG	<u>CC</u>AATTTT<u>GG</u>
oAN30	CTATAGACTATATAGTATATA**GG**<u>**AAAATT**</u>**CC** ACCACATGAACAAAAAG	5' CArG	<u>CC</u>AAAATT<u>GG</u>
oAN31	GGGAGAACTGGCACCGGAA**GG**<u>**AAATTT**</u>**CC**A GTAACCCATCGAAC	3' CArG	<u>CC</u>AAATTT<u>GG</u>
oAN32	GTTCGATGGGTTACT**GG**<u>**AAATTT**</u>**CC**TTCCGG TGCCAGTTCTCCC	3' CArG	<u>CC</u>AAATTT<u>GG</u>
oJJR47	CTATCAAATGA**T**<u>**TGTCT**</u>ACACTGTTGATTTA TTTCG	5' ARE	TGTCTC
oJJR46	CAACAGTGT<u>**AGACA**</u>**A**TCATTTGATAGGAAA ACG	5' ARE	<u>GAGAC</u>A
oJJR48	TAACCAGAAAAAAACT**T**<u>**TGTCT**</u>CATGCAAA AAG	3' ARE	T<u>GTCTC</u>
oJJR49	CATG<u>**AGACA**</u>**A**AGTTTTTTTCTGGTTAAATGC AC	3' ARE	<u>GAGAC</u>A

Table 2. Oligonucleotides Used for the Mutagenesis of Individual *FUL* Promoter Motifs

The following list of oligonucleotides were used to mutagenize each of the ARE and CArG motifs contained within the *FUL* promoter. The motif being mutated is highlighted in bold, with the altered base pairs underlined. In the columns to the right, the name of the mutated motif along with its WT sequence is given (mutated bp's underlined).

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