UNIVERSITY OF CALIFORNIA, SAN DIEGO

Expanding the genetic network controlling fruit development in *Arabidopsis thaliana*

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by

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The Thesis of Lindsay Johanna Bailey is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I dedicate this thesis to my parents and my family and lovely Golden Retriever, Casey Jane. I cannot imagine going through these past few years without all of your encouragement, love and wagging tails.

TABLE OF CONTENTS

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ABSTRACT OF THE THESIS

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The fruit is perhaps the most essential organ in *Arabidopsis thaliana*, not only because it houses and protects the developing seeds and later disperses them, but also because it is also the only link between that plant and the next generation. The majority of the *Arabidopsis thaliana* fruit is comprised of an ovary, which consists of three primary tissue types: the valves, the repla and the valve margins. Our previous studies have shown there is a complex regulatory network controlling the formation of each of these territories. The MADS-box gene *FRUITFULL* (*FUL*) is one of the most important factors regulating valve development in the *Arabidopsis* fruit. Our previous studies delimited the regulatory regions required to reproduce *FUL* endogenous expression in which several cis-regulatory motifs were identified. In this context we

wanted to further investigate the transcriptional control of the *FUL* gene by determining the importance of some of these regulatory motifs. We have found that two of the transcription factors modulating *FUL* expression in the valves are in turn, post-transcriptionally regulated by small RNAs. Collaborating with *FUL*, the *JAG/FIL* function is also required for proper valve formation. From our studies we have also identified a putative upstream microRNA-regulatory node modulating *JAG/FIL* activity in valves. We have incorporated these novel functions into our models and created a revised regulatory network controlling fruit development.

INTRODUCTION

Arabidopsis thaliana is used as a model organism

The flowering plant *Arabidopsis thaliana* (*Arabidopsis* hereafter) is a member of the Brassicaceae family (commonly known as the mustards), to which crops such as broccoli, cabbage or canola also belong. Several features such as: small size, rapid generation time (5-6 weeks), ability to grow well in controlled conditions, ease of cross-fertilization and self-fertilization, small chromosome number and ease of mutagenesis or of generating transgenic lines using *Agrobacterium*-mediate transformation (Bowman, 1993; Meyerowitz, 1989) make *Arabidopsis* the reference organism "per excellence" in Plant Biology. In addition, in 2000, *Arabidopsis* became the first plant to have its genome completely sequenced (Arabidopsis Genome, 2000). This data provided the foundation to allow for more comprehensive studies of identifying a wide range of plant-specific gene functions as well as processes that are conserved in all eukaryotes (Arabidopsis Genome, 2000). Since then, orthologous genes have been found in many crop species such as wheat (*Triticum aestivum*) (Murai et al., 2003), rice (*Oryza sativa*) (Jiao et al., 2010) or tomato (*Lycopersicom esculantum*) (Zhang et al., 2011). All of these things considered, it is no surprise *Arabidopsis* has become the reference plant for hundreds of plant biology labs across the globe.

Vegetative development of *Arabidopsis*

Flowering plants go through a succession of distinct growth phases; vegetative growth, followed by a reproductive phase and eventually seed set and senescence. The transition between these phases largely depends on the action of both environmental and endogenous stimuli that impinge upon developmental genetic networks (Huijser and Schmid, 2011).

During embryo development two populations of stem cells are established at opposing ends of the primary growth axis of the developing embryo, forming the root apical meristem (RAM) and the shoot apical meristem (SAM) (Huijser and Schmid, 2011). These meristems will give rise to all post-embryonic organs formed throughout the life of the plant. Whereas the RAM will give rise to the roots, the SAM will give rise to all above ground structures except the hypocotyl and cotyledons (Janosevic et al., 2007) (Figure 1A). Following germination, the pant passes through a stage of vegetative growth that can be divided into the juvenile and adult vegetative phases (reviewed in (Huijser and Schmid, 2011)).

The transition from juvenile to adult vegetative growth is accompanied by relatively minor changes in leaf morphology (Huijser and Schmid, 2011; Zhu and Helliwell, 2011). In the juvenile vegetative stage, the leaves are small and rounded, with long petioles, smooth margins and adaxial trichomes, while in the adult phase the leaves are larger and elongated, spatulated with shorter petioles and serrated margins and trichomes can be found on both adaxial and abaxial sides (Bowman, 1993; Huijser and Schmid, 2011) (Figure 1B). After adult vegetative growth, the plant will begin its transition from vegetative phase to reproductive phase. This is known as the floral

transition and is probably one of the most important and dramatic phase changes in plant development (Araki, 2001).

As it will later be shown, these phase transitions are tightly regulated by small RNAs (sRNAs) and their predicted target genes (reviewed in (Fornara and Coupland, 2009) and (Huijser and Schmid, 2011)). Interestingly, these sets of regulators have been designated as key controllers of the general "aging pathway" in *Arabidopsis* (Poethig, 2009; Wu et al., 2009) (reviewed in (Huijser and Schmid, 2011)).

Reproductive development of *Arabidopsis*

During the floral transition plants switch from vegetative to reproductive growth, and the SAM transforms into an inflorescence meristem, where floral meristems will develop laterally, each containing a whorled organ primordia (Ferrándiz et al., 1999; Huijser and Schmid, 2011) (Figure 1C). *Arabidopsis* contains four whorls of organs; sepals, petals, stamens and carpels. These four sets of organs comprise the flower, with the two fused carpels in the center making up the gynoecium or female part of the flower (Ferrándiz et al., 1999; Meyerowitz, 1989; Robles and Pelaz, 2005). The gynoecium can be divided into three regions from top to bottom: the stigma, style and ovary (Figure 2). The ovary conforms the majority of the gynoecium and houses the developing ovules (Roeder and Yanofsky, 2006). Three distinct tissue types can be distinguished on the outer side of the ovary: the valves, the replum and the valve margin (Figure 2). After fertilization, the gynoecium becomes a fruit and the valves rapidly increase in size enclosing and protecting the seeds as they develop. The valves are separated from each other by the replum. At the valve-replum

boundary we find a narrow strip of cells referred to as the valve margin. Considered as the ripening region in *Arabidopsis*, the valve margin is in turn sub-divided into two layers: the replum-proximal layer called the separation layer, and the valve-proximal layer called the lignified layer (Figure 2). When the fruit reaches its final length and maturity several enzymatic and mechanical processes take place at the valve margin which causes the valves to detach form the replum and release the seeds (Ferrándiz, 2002; Ferrándiz et al., 1999; Roeder and Yanofsky, 2006; Spence et al., 1996). Therefore, the correct development of all these tissues is of upmost importance for securing the seed propagation and the next generation success.

The Yanofsky lab has carried out numerous studies to better understand the genes that are required for patterning and developing the *Arabidopsis* fruit. As a result, an enormous amount of progress has been made in the past few years in identifying the key regulators of fruit development and, further, elucidating their genetic interactions. These efforts led to the construction of the current regulatory network orchestrating fruit (Alonso-Cantabrana et al., 2007; Dinneny et al., 2005; Dinneny et al., 2004; Ferrándiz et al., 2000b; Gu et al., 1988; Rajani and Sudaresan, 2001; Ripoll et al., 2011). However, our work also indicates that this research is far from over as new genes are being discovered and incorporated into these networks.

Current genetic network controlling fruit development

The formation of the valve margin is controlled by a set of regulatory genes collectively known as the valve margin identity genes (Liljegren et al., 2000; Liljegren et al., 2004; Rajani and Sundaresan, 2001). This suite of genes includes the MADS-box genes *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*) (Liljegren et

al., 2004). These two *SHP* genes work upstream and positively regulate the expression of the bHLH transcription factors *ALCATRAZ* (*ALC*) and *INDEHISCENT* (*IND*) (Liljegren et al., 2000; Liljegren et al., 2004; Rajani and Sundaresan, 2001) (Figure 3). While *IND* specifies the formation of the lignified layer of the valve margin alone, the combined activity of *IND* and *ALC* is required for proper separation layer formation (Liljegren et al., 2004; Rajani and Sundaresan, 2001). Mutations affecting these genes result in silques that fail to dehisce (Liljegren et al., 2004; Rajani and Sundaresan, 2001).

The expression of the valve margin identity genes become confined to the valve margin by the combined activities of the MADS-box gene *FRUITFULL* (*FUL*) (Gu et al., 1998) and the BEL-homeodomain gene *REPLUMLESS* (*RPL* also known as *PENNYWISE*, *BELLRINGER*, and *VAAMANA*) (Bhatt et al., 2004; Byrne et al., 2003; Smith and Hake, 2003) to prevent the ectopic expression of the valve margin identity genes in the valves and repla, respectively (Liljegren et al., 2004; Roeder et al., 2003). This regulation ensures the valve margin identity genes to be expressed specifically in the valve margin (Ferrándiz et al., 2000b; Gu et al., 1998; Roeder et al., 2003) (Figure 3). Consistent with this interaction, the valve margin genes in *ful* and *rpl* mutants become ectopically expressed in either the valve or the replum, and as a result these tissues then adopt valve margin fate (Liljegren et al., 2004; Roeder et al., 2003). Additionally, *ful* mutants result in shorter fruits due to the reduction in valve cell size, suggesting that besides its role in repressing valve margin genes, *FUL* also plays an important role in fruit valve growth.

In addition to *RPL* and *FUL*, there exists another layer of regulation controlling patterning in the fruit. The class I *KNOTTED1*-like homeobox (KNOX) gene *BREVIPEDICELLUS* (*BP*; also known as *KNAT1* (Venglat et al., 2002)) positively regulates replum formation as its over-expression results in fruits with enlarged repla (Alonso-Cantabran et al., 2007). In addition, it has been found that *BP* genetically and molecularly collaborates with *RPL* in directly regulating replum formation and together are considered the replum identity genes (Alonso-Cantabrana et al., 2007; Girin et al., 2009; Ripoll et al., 2011). Both *BP* and *RPL* and the valve margin identity genes are negatively regulated in the replum and valve margin respectively by the floral homeotic gene *APETALA2* (*AP2*) (Ripoll et al., 2011). Consistent with this regulation, *ap2* mutants have enlarged repla and valve margins (Ripoll et al., 2011).

BP is also negatively regulated in the fruits (Alonso-Cantabrana et al., 2007) (as it happens in the leaves) by the combined activities of the MYB transcription factor *ASYMMETRIC LEAVES1* (*AS1*) and the *LATERAL ORGAN BOUNDRY* (LOB) domain protein *ASYMMETRIC LEAVES2* (*AS2*) (Byrne et al., 2000; Byrne et al., 2002; Guo et al., 2008; Ori et al., 2000; Semiarti et al., 2001). Consequently, mutations in either *AS1* or *AS2* results in fruit with enlarged repla and also reduced valves (Alonso-Cantabrana et al., 2007). Besides *BP* in the replum, two other class-I KNOX family members, *KNAT2* and *KNAT6* are negatively regulated by the replum identity genes and by the valve factors (our unpublished data and (Ragni et al., 2008)).

The C2H2 Zinc-Finger transcription factor *JAGGED* (*JAG*; (Dinneny et al., 2004)) and the YABBY genes *FILAMENTOUS FLOWER* (*FIL*; (Sawa et al., 1999)) and *YABBY3* (*YAB3*; (Siegfried et al., 1999)) were initially identified for their role

during leaf development. However, recently they have also been incorporated into the regulatory network controlling fruit development. In the fruit, the activities of these genes are more commonly referred to as *JAG/FIL* function (Dinneny et al., 2005) and they have recently been shown to work together redundantly with *AS1,2* (or AS function) in a gradient fashion along the mediolateral axis of the *Arabidopsis* fruit (Alonso-Cantabrana et al., 2007; Dinneny et al., 2005; González-Reig et al., submitted) controlling the expression of valve, valve margin and replum genes. Thus, whereas high and medium levels of these activities promote *FUL* activity in the valves and the activation of the valve margin identity genes in the valve margin, low levels allow the expression of *BP* and *RPL* in the replum. As expected, when *JAG/FIL* or *AS* functions are disturbed, patterning of the fruit is altered. For instance, *fil yab* double mutants result in tiny fruits with small valves and enlarged repla, similar to *ful* fruit phenotypes (Dinneny et al., 2005; Dinneny et al., 2004).

Importance of small RNAs in plant development

Although initially conceived as "molecular garbage", non-coding RNAs are now known as key elements in the machinery involved in modulating gene expression at the post-transcriptional level. Among the non-coding RNAs, the small RNAs are perhaps the most studied. MicroRNAs (miRNAs) are short (~21-24 nucleoties) small non-coding RNA molecules that function as post-transcriptional negative regulators of gene activity (Bartel, 2004; Todesco et al., 2010). In *Arabidopsis*, miRNAs are first transcribed by RNA polymerase II in the nucleus and form a pri-miRNA, which adopts a secondary hairpin structure. The pri-miRNA is then processed to produce a dsRNA miR-segment containing a guide strand and a strand that will be degraded, and

is exported out of the nucleus into the cytoplasm (Chen, 2005; Park et al., 2005). The non-guide guide strand is degraded and loaded into a large complex of proteins forming the mature miRNA (Figure 5A). At this point, the mature miRNA is able to recognize its specific messenger RNA (mRNA) sequence, targeting it for degradation, or in some cases blocking the translation of the corresponding mRNA (Bartel, 2004).

Another family of small non-coding RNAs are the trans-acting small interfering RNAs (ta-siRNAs) (Warkocki and Figlerowicz, 2006). This group of plant specific small riboregulators are generated from *TAS* gene-derived transcripts, and similarly to miRNAs, they repress gene expression through post-transcriptional gene silencing (Felippes and Weigel, 2009). Whereas miRNAs and tasiRNAs share some common features at the functional level, their biogenesis differs. In plants, *TAS* genes messengers are long double stranded RNA transcripts which are recognized by a specific miRNA, a required recognition for site-directed cleavage of ta-siRNA transcripts, to produce many ta-siRNA duplexes (Allen et al., 2005; Axtell et al., 2006; Felippes and Weigel, 2009) (Figure 5A). The non-guide strand in the duplex is degraded and the guide strand is loaded into a complex of proteins, forming a mature siRNA, which is able to recognize its specific mRNA sequence, targeting it for degradation, similar to mature miRNAs.

Small non-coding miRNAs have been shown to be involved in many developmental processes. They affect processes such as embryonic development (Carrington and Ambros, 2003), root growth (Wang et al., 2005), leaf morphogenesis (Liu et al., 2011), phase change (Wu et al., 2009) or flowering time (Aukerman and Sakai, 2003) (Figure 5C). However, whereas small RNA biogenesis and the process of

how the resulting miRNAs and/or tasiRNAs recognize and negatively regulate their targets have been heavily researched, very little is known about the upstream layer of regulation controlling *MIR* or *TAS* encoding genes.

Our recent studies, including this work, led us to the discovery of some small non-coding RNAs that impinge upon fruit development. Our efforts now turn to incorporating these riboregulators into the current regulatory networks orchestrating fruit development. Using this developmental process, a substantial part of this work is focused on the elucidation of this upstream layer of transcriptional control using fruit development as a platform.

FRUITFULL seems to be a "regulatory-hub" for sRNA-target regulatory nodes during fruit patterning and growth

As mentioned earlier, *FUL* is a pivotal gene in fruit morphogenesis. And as expected, it is strongly expressed in the fruit. In fact, although *FUL* is endogenously expressed early in plant development, its expression becomes essentially restricted to the valve tissue of carpels by stage 8 (Ferrándiz et al., 1999) (Figure 4A,B). Interestingly, after fertilization, *FUL* adopts a bipolar expression pattern, with strong activities in the poles of the valves and little to no activity in the middle (Figure 4C). But besides its role in the fruit, *FUL* is also involved in meristem identity and flowering time (Melzer et al., 2008; Wang et al., 2009). It has recently been shown that *FUL* is a direct target of SPL transcription factors (Yamaguchi et al., 2009). The *Arabidopsis SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL)* gene family represents a group of seventeen transcription factors and most interestingly, eleven out

of the seventeen *SPL* genes contain target sites for microRNAs miR156 and miR157 (Rhoades et al., 2002). Consistently, when *MIR156* is mis-expressed, the *SPL* genes are drastically downregulated (Schwab et al., 2005; Schwarz et al., 2008) therefore affecting *FUL* activity during the control of flowering time (Kim et al., 2012).

As it will be discussed in detail in the Results section, the miR156/157-SPL regulatory node appears to be regulating *FUL* activity in the fruit, suggesting that it is conserved in both developmental processes. In addition, we have identified an additional small RNA-regulated transcriptional regulator that seems to negatively regulate *FUL* expression in valve tissue. Further, *FUL* activity is positively regulated by *JAG/FIL* activity, and our results suggest this is also under the control of an upstream small RNA regulatory node. These results indicate the regulation controlling fruit development is more complex than we previously thought and further, the regulation by transcriptional and post-transcriptional activity are tightly linked.

MATERIALS AND METHODS

Plant materials

The plant materials used in this study were: *FUL::GUS* (Nguyen, 2007); *RVSE::GUS* and *(s)RVSE::GUS* (Woods, 2010); *35S::SPL3*^{WT}*, 35S::SPL3*^{156R} and *35S::MIR156A* (Wu and Poethig, 2006); *35S::ARF3*^{WT} and *35S::ARF3*^{tasiR} (Hunter et al., 2006); *mARF10* (Liu et al., 2007); *MIR156A::GUS, MIR156B::GUS, MIR156D::GUS, MIR156E::GUS* and *MIR156F::GUS* (this work);, *FUL*$^{Aux-/-}$ (Woods, 2010); *JAG::GUS* (gift from José Dinneny), *ARF3::GUS* and *MIR390::GUS* (Chitwood et al., 2009), and the *FUL::LhG4* driver line was created in our lab. All the lines were obtained in the Columbia (Col-0) background.

Cloning strategies used in this work

RVSE::GUS and *(s)RVSE::GUS* have been previously described in (Woods, 2010). To create transcriptional *β-glucuronidase* (*GUS) MIR156* reporters, the promoter fragments of *MIRNA156A* (AT2G25095), *MIRNA156B* (AT4G30972), *MIRNA156D* (AT5G10945), *MIRNA156E* (AT5G11977) and *MIRNA156F* (AT5H26147) genes were each amplified using a proof-reading, high-fidelity Taq Polymerase (Phusion from New England Biolabs) using Col-0 genomic DNA as a template. The putative 5' regulatory sequence of *MIR156A, MIR156B, MIR156D, MIR156E,* and *MIR156F* (primers in Table 1) were cloned into the *GUS* gene reporter of the pJJGUS T-DNA vector (Ripoll et al., 2006) using the restriction sites included in the corresponding primers (Table 1).

For generating the *OP::MIM156* construct the primers oJJR267 and oJJR268 were used to amplify the *MIM* region from the pGem-T (Promega, Inc.) precursor vector (Todesco et al., 2010) and cloned into the pBJ3-10xOP via *Kpn*I and *BamH*I sites. After checking the integrity of the sequence, the 10xOPMIM156 cassette was excised using the *Not*I sites and cloned into the T-DNA binary vector pGreenII0179 (Hellens et al., 2000).

To generate the pLacZi constructs to do the Y1H assays, primers were designed on the *MIR390A* (AT2G38225) promoter for every 500bp with 20bp overlapping for each fragment (see Table 3). Each fragment was amplified using Phusion Taq polymerase (NEB) and cloned into the pLacZi (Clontech vector) using classical cloning techniques.

Creation and selection of transgenic plants

Each pGreen T-DNA resulting construct was co-transformed with the helper vector pSOUP (Hellens et al., 2000) into *Agrobacterium tumefaciens* (AGL0 or LBA4404 strain) via electroporation. For plant transformation we used Col-0 background *Arabidopsis thaliana* plants and followed the floral dipping method (Clough and Bent, 1998) and used Col-0. T1 transgenic plants for *OP::MIM156*, *MIR156A, MIR156B, MIR156D, MIR156E* and *MIR156F* were selected by sowing seeds on MS plates containing 20mg/ml Hygromicin and transplanted to soil after 2 weeks. F1 plants for *FUL>>OP::MIM156* underwent double selection using 20mg/ml Hygromicin plates and subsequent spraying with BASTA (120 mg/ml ammonium glufosinate; Finale, AgrEvo, Montvale, NJ), twice a week for three weeks.

GUS staining

Inflorescence, seedlings, and fruit tissues were treated as previously described (Ripoll et al., 2011). A standard dissecting scope with an adapted camera was used to take tissue whole-mount pictures.

SEM Microscopy

Scanning electron microscopy (SEM) was preformed as previously described (Ripoll et al., 2006).

In situ hybridizations

Messenger RNA *in situ* hybridizations were preformed as described before (Dinneny et al., 2004; Dinneny et al., 2006).

Yeast transformation

Each pLacZi resulting construct was initially with *Nco*I and transformed into yeast strain YM4271 as follows. To prepare the competent yeast cells, a 50ml culture of YM4271 was grown overnight in YPD at 30°C with shaking. 5ml of the overnight culture was added to 150ml of YPD and incubated at 30°C until the OD_{600} reached 0.4-0.6. 50ml of the new culture was then centrifuged at 1,000xg for 5 minutes, and washed three times with sterile milli-Q water. The cell pellet was then resuspended in 1.5ml of fresh 1xTE (10 mM Tris pH 7.5, 0.05 mM EDTA) /1xLiAc (pH 7.5). We added 0.1 mg of single stranded carrier salmon sperm DNA to 100µl of the resulting

competent yeast. To each tube, 0.6ml of sterile PEG (50% PEG 3,350)/1xTE/ 1xLiAc was added to each tube and incubated at 30°C for 30min with gentle shaking. We then added 70µl of DMSO was added to each transformation and mixed by gentle inversion. The tubes were heat shocked for 15 minutes at 42°C and then iced for 2 minutes before being centrifuged for 10 seconds at max speed. After removing the supernatant, the pellet was resuspended in 150µl 1xTE, and plated on SD –ura. The plates were incubated at 30°C in a humidified box for 3 days. Colonies were then checked by PCR.

Yeast one-hybrid

The Y1H assays were preformed by José Pruneda-Paz as previously described (Pruneda-Paz et al., 2009).

Transient-Assay in Tobacco

The transient expression in tobacco assay was preformed following the directions of Guan et al. (unpublished) with minor modifications.

RESULTS

As mentioned earlier in the Introduction, *FUL* plays pivotal roles not only in fruit patterning and growth (Ferrándiz et al., 2000b; Gu et al., 1998) but also in controlling meristem identity and flowering (Melzer et al., 2008; Wang et al., 2009). The correct transcriptional control of regulatory genes, like *FUL*, has been described as critical in many plant and animal species as it is key to achieve proper development. Therefore our group decided to functionally dissect the regulatory regions of the *FUL* gene in order to elucidate the transcriptional elements controlling *FUL* expression.

Former members of our research group determined that the 3.9 kb genomic region up-stream of the ATG of the *FUL* gene was sufficient to recapitulate the endogenous expression of *FUL* and, moreover, capable of rescuing *ful* mutants (Nguyen, 2007; Woods, 2010). They preformed promoter bashing and *in silico* analysis on the 3.9 kb promoter region of *FUL* and identified six main functional cis-regulatory motifs located within the promoter region. These included two CArG boxes, two Auxin Responding Elements (AuxREs) and two SPL binding sites (Woods, 2010) (Figure 4D). In addition, the promoter bashing analysis led to the identification of a promoter fragment which when used in transgenic *GUS*-reporter lines, exclusively showed carpeioid valve expression (Woods, 2010) (Figure 6B). This region was referred to as *RVSE* (*Region of Specific Valve Expression*) and contains one of the two CArG-boxes and the 5' *SQUAMOSA*-related binding cis-motif (Figure 6A).

I.1. SPL domain is required for regulating *FUL* transcription levels in carpels and valves

As previously mentioned, the *Arabidopsis SPL* gene family represents a group of seventeen transcription factors that share a common *SQUAMOSA*-promoter-binding protein domain (SPB) (Cardon et al., 1999). This highly conserved domain includes a zinc finger motif that functions as the DNA-binding domain (Yamasaki et al., 2004). These transcription factors have been shown to be involved in different developmental processes such as leaf development, flowering time regulation and flower development (Schwarz et al., 2008; Wang et al., 2009). Interestingly, these transcription factors were among the first transcriptional regulators shown to be miRNA-regulated in *Arabidopsis* (Rhoades et al., 2002; Unte et al., 2003).

Two independent works recently show that SPL3 directly activates *FUL* expression by binding to the 3' SPL cis-motif of the promoter (Wu et al., 2009; Yamaguchi et al., 2009). Because of this direct activation, we wanted to investigate the role of the SPL binding site located within the *RVSE* fragment of the *FUL* promoter. We thus compared the resulting expression patterns in *RVSE::GUS* lines to that of transgenic plants harboring *(s)RVSE::GUS* constructs. The *(s)RVSE::GUS* promoter fragment lacks a 119 bp DNA piece that contains the 5' SPL binding site (Woods, 2010). Whereas in the gynoecium and fruits from the *RVSE::GUS* plants we were able to detect strong and homogeneous *GUS* activity in the valves (Figure 6B), in *(s)RVSE::GUS* transgenics, although the expression was still valve-specific, the transcription levels were drastically reduced (Figure 6C). This strongly suggests that

the 5' SPL binding motif within the *RVSE* region plays a significant role in activating *FUL* transcription in carpels and valves.

I.2. *SPL3* activates *FUL* promoter in carpel/valve tissue in a miR156-dependent manner

The fact that at least one of the SPL (SPL3) transcription factors was found to directly regulate *FUL* expression in the developmental context of floral transition (Cardon et al., 1997; Wu and Poethig, 2006) led us to study whether this same regulation was also taking place in the fruit valves. To do these experiments we crossed our transgenic reporter line *FUL::GUS* to *35S::SPL3*^{WT}. We tested the behavior of the GUS reporter in the fruits of the resulting F1 plants in which no difference in levels or expression pattern was detected when compared to our control line *FUL::GUS* (Figure 7A,B). However, because we were aware that *SPL3* is miR156-regulated, we tested the *FUL::GUS* activity in plants misexpressing a miR156-immune version of SPL3 (*35S::SPL3*^{miR156R}) . As can be easily depicted from Figure 7C, in *35S::SPL3*^{miR156R} fruits the levels of *FUL::GUS* activity were up-regulated, but the *FUL*-reporter still displayed the typical bipolar pattern. This observation suggests that SPL3 (likely in a direct manner) is capable of activating *FUL* transcription in fruit valves. But, however this regulation is miR156-dependent since no up-regulation was observed in *35S::SPL3*^{WT} (Figure 7B,C).

We then wondered if a fruit phenotype results from the overexpression of miR156, since it is likely that it is active in the fruit. We expect to see similar fruit phenotypes to *ful* mutants, with miR156 excessively repressing the *SPLs* in the valves

therefore limiting *FUL* activation, and indeed we do. *35S::MIR156A* reporters result in short fruits with tailored valves, similar to *ful-2* (Figure 7D). This is a direct result of reduction in valve cell size. The valve cells, instead of being slender and elongated like seen in WT (Figure 7E), are much shorter and stumpy (Figure 7F). This observation leads us to believe that *MIR156* is playing an important role in regulating fruit morphogenesis, acting through the *SPLs* to help regulate *FUL*. It has been reported that miR156 is present in both reproductive and vegetative tissues (Gandikota et al., 2007; Wu and Poethig, 2006; Xing et al., 2010) however *MIR156* expression patterns have not yet been carefully examined. We expect that at least one member of *MIR156* is active in the fruit, specifically the valves; therefore we decided to carefully evaluate *MIR156* expression in the fruit tissues as well as in seedlings and roots.

I.3. Analysis of the expression patterns of *MIR156* encoding genes in *Arabidopsis thaliana*

We decided to use a strategy that has been successfully used before for other MIR families in *Arabidopsis* and other species to examine *MIR* expression patterns (Chitwood et al., 2009; Mai, 2009; Miura et al., 2010; Warthmann et al., 2008; Xing et al., 2010). Thus, we generated transgenic plants harboring *GUS*-based reporter lines for five of the eight *MIR156* encoding-loci (*MIR156A::GUS, MIR156B::GUS, MIR156D::GUS, MIR156E::GUS,* and *MIR156F::GUS*) and examined their corresponding expression patterns. We analyzed the expression patterns for each reporter in several T1 plants and verified in the next generation. Using these

transgenic plants we generated a map of expression in seedlings, inflorescences and carpel/fruits for each *MIR156 GUS*-reporter.

Because previous studies have only analyzed the expression of *MIR156* in anthers (Xing et al., 2010), we decided to carefully study the resulting patterns of our *GUS*-reporters in flowers and fruits. *MIR156A::GUS* constructs displayed moderate levels of homogenous expression in the valves, high expression in the stigma and no expression in the style. *MIR156B::GUS* constructs showed no activity in the fruits, but very faint activity can been detected in the anthers (not shown). Reporters for *MIR156D::GUS* and *MIR156E::GUS* displayed very similar expression patterns in the fruit; high levels of *GUS* expression were detected in the valves, replum and stigma, and no expression in the style (Figure 8D,E). Reporters harboring *MIR156F::GUS* displayed low levels of expression in the carpels. The valves, replum and stigma all displayed low levels of expression, no expression in the style and high expression in the carpel vasculature (Figure 8F). These observations help solidify our hypothesis that miR156 is acting through the SPL transcription factors to regulate the expression of *FUL* in fruits.

It has been reported that *MIR156* plays an important role in regulating the juvenile phase in vegetative tissue, but the expression patterns of *MIR156* has yet to be carefully examined in vegetative tissue. Using the same constructs as mentioned previously, we analyzed the expression patterns of our *GUS*-reporters in 12-day-old seedlings. All reporters showed activity in the roots, but not all were active in the leaves (Figure 7 and data not shown). For *MIR156B::GUS, MIR156E::GUS* and *MIR156F::GUS* no expression was detected in the lateral organs during vegetative

development. However, medium and high levels of expression were detected in the leaves of *MIR156A::GUS* and *MIR156D::GUS* (Figure 8F,G). Interestingly, some of the miR156-regulated *SPL* genes are active in leaves and, whereas their transcriptional constructs are highly expressed in these tissues, translational reporters show lower activities suggesting that miR156 might be regulating *SPL* function in the leaves.

I.4. Alteration of *miR156* levels disrupts normal lateral organ development

The *MIR156* expression patterns in the leaves indicate that *miR156* is present in vegetative tissue. This result is consistent with previous studies preformed on *MIR156* reporting that it is highly expressed early in shoot development (Wu et al., 2009). Because miR156 acts on the SPLs, we wanted to research the involvement of the SPLs in leaf and fruit development. Loss-of-function *SPL3* mutations generally have no leaf phenotype but the constitutive expression causes early flowering (Wu et al., 2009) indicating SPLs are present and active in vegetative tissue. Plants expressing *MIR156A* under the regulation of the constitutive 35S promoter produce a large amount of juvenile leaves and has an extremely late flowering time, whereas plants expressing a nucleic acid sequence able to sequester naturally occurring miR156 (*35S::MIM156*) produce adult leaves almost immediately and flower early (Wu et al., 2009; our unpublished data). The first two rosette leaves in *35S::MIM156* plants are unusually large and elongated, with serrated leaf margins and abaxial trichomes, with the later formed leaves being larger but nearly identical in shape to the first two rosette leaves (Wu et al., 2009). These results are consistent with the results suggesting *MIR156* is expression in vegetative tissue. However, we are most interested in the

effect miR156 has on *FUL* expression, so using the two component system we decided to express *MIM156* exclusively in regions where *FUL* is expressed.

This strategy has been successfully used before in *Arabidopsis* (Baroux et al., 2005; Brand et al., 2006) and other plant species (Fernandez et al., 2009) and is a reliable way to induce mutant phenotypes using conditional ectopic gene expression. Thus we generated a transgenic inducible line *OP::MIM156* (see Material and Methods) and crossed the resulting T1 with the driver line in which the *LhG4* transgene was under the control of the *FUL* promoter (*FUL::LhG4*). We expected to see a phenotype in the leaves only where *FUL* is active (Figure 9A). We observed a very mild phenotype for the resulting F1's. The *FUL>>MIM156* lines were slightly smaller than the control plants (*OP::MIM156*) (Figure 9B,C). The petioles are shorter and the leaves are slightly more jagged, reminiscent of *jag* mutants (Figure 9D,E). We also noticed that the fruits of *FUL>>MIM156* were shorter with more tailored valves than WT but not as short as *35S::MIM156* (results not shown).

II.1. AuxRE's re involved in generating the bipolar expression pattern of *FUL* in fruit valves

In addition to the SPL binding domains and CArG-boxes, the *FUL* promoter contains two canonical AuxRE (TGTCTC) cis-regulatory motifs located at -367 bp and -641 bp upstream of the *FUL* start codon (Figure 10A) (Woods, 2010). The AuxRE motifs are recognized by the extended family of AUXIN RESPONSE FACTORS (ARFs), which can activate or repress target gene expression (Tiwari et al., 2003).

During gynoecium development *FUL::GUS* expression is homogeneous in carpel tissue, but at the anthesis stage, and thus fertilization of the pistil, the *GUS* expression levels reduce in the middle region of the valves which create the bipolar expression pattern of *FUL* in the valves (Figure 10C). However, a former student in Professor Yanofsky's lab found that in *GUS*-reporters in which both AuxREs were mutagenized (*FUL*$^{AuxRE-/-}$*::GUS*) the typical bipolar pattern was not achieved and instead a homogenous expression pattern was observed (Woods, 2010) (Figure 10B), suggesting that the AuxREs help mediate the repression of *FUL* in the middle region of the valve.

At that point the key was to identify which *ARF* was responsible for the repression of *FUL* expression in the medial area of the valves. Because of our preliminary data, our best candidate for an ARF capable of repressing *FUL* expression in the middle of the valve was ARF3 (aka *ETT*; Fahlgren et al., 2006; Hunter et al., 2006; Sessions and Zambryski, 1995; Yant, 2012). Interestingly ARF3 is post-transcriptionally regulated by a subset of miR390-dependent TAS3-derived ta-siRNAs (trans-acting short-interfering RNAs) termed tasiARFs (Williams, 2005 #1201).

II.2. Correct fruit development and growth requires tasiARF-regulated ARF3

Although the regulation of *ARF3* by tasiARFs was initially identified to be critical in regulating leaf polarity in *Arabidopsis*, recent works show that it also plays an important role in lateral root growth (Marin et al., 2010) and in modulating the developmental timing of the plant (Fahlgren et al., 2006; Hunter et al., 2006). However no specific role has been previously assigned to this tasiARF-ARF3

regulatory node during fruit growth and development. Interestingly it has been proven that these siRNA molecules are able to travel a short distance from where they are made (Felippes et al., 2010; Melnyk et al., 2011), likely creating a gradient of activity (Chitwood and Timmermans, 2010).

To determine the importance of this regulation in the context of fruit, we first characterized the pistils of transgenic lines mis-expressing a tasiARF-immune version of ARF3 cDNA (*35S::ARF3*^{tasiR}) and compared the resulting fruits to those of *35S::ARF3*^{wt} and wild-type plants. No obvious fruit alterations were detected in *35S::ARF3*^{wt} (our unpublished data, not shown). However *35S::ARF3*^{tasiR} fruits showed dramatic reduction in fruit length even after hand pollination, as it occurs in *ful-2* mutants. We also observed defects in hemizygous *35S::ARF3*^{tasiR} (*35S::ARF3*$^{tasiR/+}$hereafter) fruits. In these lines fruits were smaller (around 60% shorter) when compared to wild-type. Also the valves were more tailored to the seeds, which gave the siliques a bumpy appearance. These phenotypes were reminiscent to those described before for *ful-6* (results not shown).

II.3. ARF3 is limited to the middle region of the valve by the activity of miR390/*TAS3*-tasiARF

Biogenesis of tasiRNAs have been detailed in the Introduction section. The generation of tasiARFs implies the miRNA-direct cleavage of *TAS3* messenger by miR390 (Hunter et al., 2006; Marin et al., 2010; Williams et al., 2005) in a 21-bp tailored mode that leads to the formation of the tasiARFs. These small riboregulators

in turn target and degrade *ARF3* mRNA (Fahlgren et al., 2006) and are believed to do so in a gradient fashion (Chitwood et al., 2009).

We then decided to determine the expression pattern of *ARF3*, *MIR390* and *TAS3* encoding genes in the valves to observe where tasiARFs are made and know where the *ARF3* messenger is present in order to predict where the ARF3 protein is accumulating. Transcriptional *GUS*-reporter lines for *MIR390*, *TAS3* and *ARF3* were created or obtained from other groups (see Materials and Methods) and *GUS* activity was tested in stage 15 fruits following our standard protocol (see Materials and Methods). In fruit valves the *ARF3::GUS* transcriptional construct shows homogenous distribution of the reporter signal (Figure 11C). But we strikingly found that both *MIR390::GUS* and *TAS3::GUS* showed similar expression patterns. They both displayed more activity in the poles of the valves (bottom and top) and no activity in the middle region, a pattern that was remarkably similar to that of *FUL::GUS* (Figure 11A,B,D).

Using all this data, we generated a working model in which miR390 is able to process *TAS3* messenger in the top and bottom regions of the fruit valves to allow the formation of functional tasiARFs to degrade *ARF3* messenger in the poles and, gradually, restrict its activity to the middle of the valve where ARF3 accumulates and represses *FUL* expression (Figure 11E). In fact, we have recently observed that in *35S::ARF3*$^{tasiR/+}$ fruits *GUS*-activity of *FUL::GUS* drastically dripped, even at the top and bottom parts of the valves which are regions in wild-type fruits where *FUL* is highly expressed (results not shown). This data supports the model we proposed in Figure 11E.

II.4. Identification of upstream transcriptional regulators controlling MIR390 expression in fruits

As described above, formation of the bipolar pattern for *MIR390* and *TAS3* in fruit valves triggers the accumulation of tasiARFs in the poles of the valves, limiting ARF3 protein to the middle region, which, in turn likely transcriptionally represses *FUL* expression. Therefore, one of our goals was to elucidate the upstream layer of transcriptional regulation orchestrating *MIR390* (and *TAS3*) expression pattern in fruit valves. For this purpose, we decided to undertake a high-throughput strategy passed on a yeast one-hybrid (Y1H) methodology performed using a robotic platform and an approached termed "Promoter Hiking" (developed by J. Pruneda-Paz and Steve Kay groups; Pruneda-Paz et al., 2009). This new technology uses activation of a downstream reporter gene (β-*galactosidase*, *LacZ*) as a readout of promoter binding (Deplancke et al., 2004).

We broke down the *MIR390* promoter into 5 fragments (with about a 100 bp overlapping region between the fragments) and generated the corresponding constructs using the pLacZi (Clontech) binary vector. After checking the integrity of these constructs, we transformed them into the yeast strain YM4271 (see Materials and Methods). The resulting yeast promoter strains (*yMIR390-1, -2, -3, -4, -5* respectively) were mated with each yeast line (Mav103) harboring a transcription factor in the library, and a subsequent *LacZ* assay was preformed (Figure 12A).

Our next step was to filter the resulting data. We only picked the transcription factors that were able to activate the promoter (and therefore increase *LacZ* activity)

over 4-fold when compared to our controls to corroborate the results and perform the follow up experiments. Since our concern focuses on fruit development, we sorted through the list of candidates based on their expression levels in flowers and floral organs using publicly available microarray data and several web-based programs (AtGenExpress, (Schmid et al., 2005); Genevestigator, (Zimmermann et al., 2004)) to narrow down our list of potential candidates (Figure 12B). This part of my research was done in collaboration with another MS student in the Yanofsky group (Scott Wu).

Strikingly, one of the transcriptional regulators that came out from these filters was *YAB3* (AT4G00180), a member of the YABBY gene family (Siegfried et al., 1999). Interestingly *YAB3* has been involved in specifying leaf polarity (Kumaran et al., 2002) and along with *FIL*, *JAG* and *AS1* is involved in promoting valve fate in patterning of the *Arabidopsis* fruit (Alonso-Cantabrana et al., 2007; Dinneny et al., 2005; Dinneny et al., 2004). In our Y1H assays, *YAB3* was found in 3 of the 5 fragments we tested (*yMIR390-1, -2, -3*).

At this point we wanted to test this interaction, and decided to use an *in planta* transiently based assay. Prof. Nigel Crawford's group (UCSD) has been successfully using *Nicotiana benthamia* leaves to preform these assays called TAT (Transient-Assay in Tobacco). Essentially, this technique allows us to determine in a plant cell whether a putative transcription factor is able to positively or negatively regulate a promoter using the activity of a *GUS*-reporter gene under the control of such promoter as a readout (Guan et al., unpublished).

Agrobacterium cultures were grown, one containing the *MIR390::GUS* construct and the other the *35S::YAB3*. In our first set of experiments we infected

Nicotiana leaves with *MIR390::GUS* and determined the levels of GUS activity after protein extraction and *in vitro* GUS assay (O.D. was measured at 425 nm). Our second set of experiments involved the coinfection of tobacco leaves using both *MIR390::GUS + 35S::YAB3* and the subsequent measurement of GUS activity (Figure 12C). Two biological replicates and two technical replicates were performed per each set of experiments (see Table 3). As depicted from Figure 12D, *35S::YAB3* is able to increase the *GUS*-activity levels of *MIR390::GUS*, strongly suggesting that YAB3 might act as a positive regulator of *MIR390* expression.

III.1. Correct fruit development and growth requires proper regulation of ARF10 by miR160

Our results show the importance of miR-regulation in fruit. Therefore we wanted to identify and explore additional miR-target regulatory nodes impinging upon fruit development and growth. We decided to then search through the published literature and find possible candidates to be further investigated. One of such candidates was miR160. This small riboregulator targets and degrades the messenger of three ARF genes, *ARF10*, *ARF16* and *ARF17* (Mallory et al., 2005; Rhoades et al., 2002; Wang et al., 2005). Whereas the role of the miR160-ARF regulatory node as been vigorously analyzed during leaf development (Liu et al., 2011; Mallory et al., 2005), root cap formation (Wang et al., 2005) and seed germination and post-germination (Liu et al., 2007), no comprehensive study has been preformed to elucidate its role in fruit development until this work.

We first decided to dissect the phenotypes of plants harboring a transgenic construct in which a miR160-resistant form of *ARF10* was expressed from the endogenous *ARF10* promoter (Liu et al., 2007). Hemizygous plants for this transgene (*mARF10/+* hereafter) exhibited, as previously reported, smaller and serrated leaves when compared to those of wild-type (Liu et al., 2007) (Figure 14B,C). This phenotype is reminiscent to that of *as1* (or *as2*) mutants (Xu et al., 2003) or of backgrounds in which *JAG/FIL* genes mutate (Dinneny et al., 2005; Dinneny et al., 2004; Ohno et al., 2004) (results not shown) which suggested to us some sort of (at least) genetic connection between miR160-ARF10, *AS1* and *JAG/FIL*. Dinneny et al. (2004), Alonso-Cantabrana et al. (2007) and our unpublished data suggest that JAG/FIL function and the AS genes belong to a common regulatory network controlling leaf and gynoecium development. Thus, we next carefully characterized *mARF10/+* gynoecia/fruit phenotypes.

We compared fruits of *mARF10/+* and Col, and found that the mutant displayed altered silques with a bumpy appearance (Figure 13A). A closer inspection of these fruits revealed the presence of smaller valves and an enlarged replum (Figure 13C-E) that contained more cells in the epidermal layer than those of wild type (Figure 13F,G). Again these defects were very similar to those described before for *as1, jag/fil* mutants or for *ful-6*, a weak mutant allele for the *FUL* gene (Alonso-Cantabrana et al., 2007; Dinneny et al., 2004) (Figure 13A, Figure 7D). Both fruit and leaf defects were stronger in homozygous *mARF10* plants (Figure 13A, Figure 14B,C).

ARF10, as well as ARF17 and ARF16, have been previously postulated to work as transcriptional repressors of auxin-regulated genes (Chapman and Estelle, 2009). Thus, we decided to test the expression patterns of the valve genes *JAG*, *FIL* and AS1 in *mARF10* related backgrounds. Interestingly, according to the real time PCR assays presented by Liu et al. (2011), the miR160-targeted *ARF16* gene negatively regulates *FIL*. Therefore we decided to test first the expression pattern of *FIL* in *mARF10*/+ and *mARF10* gynoecia.

We preformed an mRNA in situ hybridization on wild type, *mARF10*/+ and *mARF10* inflorescences using a *FIL* anti-sense probe. We detected strong *FIL* expression in the valves of stage 8 wild-type gynoecia (Figure 13C), slightly less expression in the valves of stage 9 *mARF10*/+ (Figure 13D) and drastic reduction of *FIL* expression in the valves of stage 9 *mARF10* gynoecia (Figure 13E). These results suggest *ARF10* is working upstream of *FIL* negatively regulating its expression.

Next we decided to check whether the expression of *JAG* was also affected by *mARF10*. To do this experiment we crossed a transgenic reporter line *JAG::GUS* to *mARF10*/+ and observed the behavior of the *GUS* reporter in the fruits of the resulting plants. Currently, we are waiting for the results for the F2 population. By checking the expression of *JAG::GUS* in the *mARF10/+* background, we will be able to see if there is an effect on the expression of *JAG* in the valves. If an effect does occur, we can combine this and previous data to suggest additional downstream targets of miR160-ARF10 activity. Further experiments can also be done to test the downstream targets of this miRNA regulatory node in fruit tissues.

DISCUSSION

To further investigate fruit morphogenesis we carried out a comprehensive analysis of the transcriptional regulation of the gene *FRUITFULL* (*FUL*). These studies lead us to the identification of several upstream transcriptional regulators required for the proper expression of *FUL* in valves. Interestingly, two of these transcription factors are post-transcriptionally miRNA-regulated. Although on one had we have expanded our regulatory network governing fruit development, our studies suggest a tight link between both the transcriptional and the post-transcriptional level of regulation.

I.1. miR156/157-SPL node regulates *FUL* expression in valve tissue

Previous works preformed in our lab led to the identification of a region within the *FUL* promoter required for carpel and valve expression, which we referred to as the *Region of Valve Specific Expression* (*RVSE*). A CArG-box and an SPL binding motif were found within this region. They both seem to be required for activating *FUL* in carpel and valve tissues. Our previous research showed the CArG-boxes are important as *RVSE::GUS* constructs lacking the CArG-box fail to activate *GUS* expression in the valves or carpels. We determined the importance of the SPL related motif by first generating a deleted version of the *RVSE* promoter termed *(s)RVSE::GUS*. Whereas both *RVSE::GUS* and *(s)RVSE::GUS* showed signal specifically in valve tissue, the levels of expression were drastically different (Figure

6B,C). In *(s)RVSE::GUS* the *GUS*-activity in the valves was, although perceptible, very low and almost absent in fruits at stage 16-17.

The fact that this SPL binding motif is necessary to achieve the correct levels of *FUL* activity in the valves made us wonder whether any of the SPL transcription factors was actually regulating *FUL* activity in fruits. It has recently been found that SPL3 is a direct upstream positive regulator of *FUL* expression in the meristem during flowering time (Wu et al., 2009; Yamaguchi et al., 2009). *SPL3* is one of the members of the SPL transcription factor family that is post-transcriptionally regulated by miR156/157, so we tested *FUL::GUS* in *35S::SPL3*^{WT} and in *35S::SPL3*^{miR156R} backgrounds. This result indicated that, in fact, the SPLs are able to activate the *FUL* promoter, and it does so in a miR156/157 dependent manner. This was not a surprise since our expression analysis revealed that the transgenic *MIR156A::GUS, MIR156D::GUS, MIR156E::GUS* and *MIR156F::GUS* reporters show strong expression in carpel and valve tissues (Figure 8B-E). Taken together, it is likely that this miR156/157-SPL regulatory node acts upon *FUL* during fruit morphogenesis.

As previously described, besides its role in fruit patterning, *FUL* seems to be also playing a role in controlling valve growth and maturation. These two fruit-related processes may be included as a part of the so-called aging pathway of plants, as fruit development leads to the end of the life cycle. It is interesting that the same miRNA regulatory pathway controls *FUL* during flowering time (aging pathway; Wang et al., 2009) and fruit development, indicating that this miR-regulatory control is crucial for the progression of the correct progression of the life cycle of the plant.

I.2. miR156/157-SPL regulatory node is likely involved in regulating leaf morphogenesis

As mentioned in the Results section, we also detected *GUS* activity in leaves of many of our *MIR156 GUS*-reporters. This was not surprising since some of the miR156-targeted *SPL* genes are active in leaves, and although their transcriptional constructs are highly expressed in vegetative tissues, translational reporters for these genes show lower activity (Yamaguchi et al., 2009), suggesting that some post-transcriptional regulation is occurring in vegetative tissue. In fact, when miR156 regulation was disrupted in *35S::MIR156* and *35S::MIM156* backgrounds, leaf development was affected (this work, Franco-Zorrilla et al., 2007; Kim et al., 2003; Wu et al., 2009). For example in *35S::MIM156* plants, leaves were more narrow, slightly more jagged and had shorter petioles than those of wild-type Col (Wu et al., 2009). It is likely that the miR156/157-SPL regulatory node plays a role during leaf development.

Interestingly, our *FUL::GUS* reporter was also active in rosette leaves. But so far no phenotype has been reporter for rosette leaves in loss-of-function mutants (Ferrándiz et al., 2000a; Gu et al., 1998). We generated a *FUL::LhGAL4* transgenic line that was crossed to *OP::MIM156*. The resulting F1 and F2 *FUL>>MIM156* plants made leaves similar to those of *35S::MIM156* (Figure 9B-E). we have recently seen that transgenic plants misexpressing *FUL* (*35S::FUL*) bear leaves with longer petioles (Ferrándiz et al., 2000a) (data not shown) similar to that of *35S::MIM156* or *FUL>>MIM156*. Although we are aware that more experiments need to be done, it is

tempting to hypothesize in this scenario that the miR156/157-SPL node might also be regulating *FUL* during leaf development.

II.1. miR390-TAS3-ARF3 regulatory module likely mediates the repression of *FUL* in the middle region of the fruit valves

In addition to the CArG-boxes and SPL binding sites, which appear to be important in regulating *FUL* expression levels in the fruits, we determined the AuxREs are also required for regulating *FUL* expression in the fruits. Whereas *FUL::GUS* fruits show bipolar *GUS* expression in the valves after fertilization (Figure 10C), $FUL^{AuxRE-/-}$*::GUS* fruits show strong homogenous expression in the valves (Figure 10B), indicating the AuxRE sites are able to repress *FUL* activity in the middle of the valves. This then made us wonder which ARF or ARFs was mediating this repression.

ARF3 is active in valve tissues (Figure 11C) and is post-transcriptionally regulated by small RNAs in other tissues (Fahlgren et al., 2006; Hunter et al., 2006; Marin et al., 2010). The tasiARF that post-transcriptionally regulates *ARF3* activity is the product of the combined activities of *TAS3* and miR390 (Hunter et al., 2006; Marin et al., 2010; Williams et al., 2005). Therefore we can extrapolate that the tasiARF is active only in the poles of the valves due to the bipolar expression of both *MIR390::GUS* and *TAS3::GUS* (Figure 11A,B), restricting *ARF3* activity to the middle of the valve where it negatively regulates *FUL* expression. Consistent with this regulation, the misexpression of *ARF3* using a tasiARF resistant version, results in short fruits with small valve cells, reminiscent to those of *ful-6* mutants (our

unpublished data). Taken together, it is likely that this miR390-TAS3-ARF3 regulatory module plays an important role in restricting *FUL* activity to the poles of the valves.

II.2. *YAB3* is a possible upstream regulator of *MIR390* in valves

We wanted to continue our research to search further upstream and attempt to elucidate any genes that may be triggering the expression of *MIR390* in the valves. Using a yeast one-hybrid approach we were able to screen the *MIR390* promoter with thousands of transcription factors found in the *Arabidopsis* genome (Pruneda-Paz et al., 2009). The list of candidates we obtained however needed a filter to increase the likelihood of the TF-*MIR390* interaction occurring in our tissue of interest. Therefore we used publically available microarray data (AtGenExpress and Genevestigator) and selected for transcription factors that were expressed in flowers and fruits (details in (Wu, 2012)). One candidate that had a high fold of induction in the Y1H and was present in flower and floral organs based on microarrays was *YAB3*.

We then tested this YAB3-*MIR390* recognition in a plant system using a TAT as described in the Results section. This allowed us to not only test if there is YAB3-*MIR390* recognition, but if there is regulation, whether it is able to activate or repress the *MIR390* promoter. We observed a two-fold increase in *GUS* expression in tobacco leaves that were coinfected with *MIR390::GUS* and *35S::YAB3 Agrobacterium* strains compared to leaves infected with only *MIR390::GUS* (Figure 12D), which strongly suggests that YAB3 is able to positively regulate the expression of *MIR390* in fruit tissue.

III.1. miR160-ARF10 node negatively regulated *FIL* expression in the valves

In addition to the MIR390-TAS3-ARF3 module, which we believe is negatively regulating *FUL* expression in the valves, our data suggests that there is yet another miR-ARF node regulating fruit morphogenesis and growth. This is the regulation by miR160 and its target genes *ARF10, 16* and *17.* This regulatory node has been analyzed in many *Arabidopsis* developmental processes previously (Liu et al., 2007; Liu et al., 2011; Mallory et al., 2005; Wang et al., 2005), however until now, no work has been published on its role in fruit morphogenesis.

The vegetative and fruit phenotypes of *mARF10/+* and *mARF10* mutants (Figure 13A, 14B,C) were reminiscent of plants in which the *AS* genes or *JAG/FIL* genes had been mutated, which suggests that these genes may all belong to a common network involved in regulating leaf and gynoecium development. More interestingly, in situ hybridization of *mARF10/+* and *mARF10* gynoecia using a reliable *FIL* antisense probe, showed a slight decrease in *FIL* activity in *mARF10/+* gynoecia and a dramatic decrease in *FIL* activity in carpeloid valve tissue *mARF10* gynoecium when compared to wild-type (Figure 13 C-E) suggesting *ARF10* is able to negatively regulate the expression of *FIL*. Because *FIL* and *YAB3* are closely related in expression pattern and sequence (Siegfried et al., 1999), we can expand this model to include *YAB3* as an additional target of *ARF10* regulation.

Additionally, *mARF10/+* and *mARF10* fruits appear to have larger repla and smaller valves than wild-type (Figure 13F,G), a defect also seen in *AS* and *JAG/FIL* mutants. Although this is only preliminary data, we suspect miR160 is able to post-

transcriptionally negatively regulate *ARF10*, and that ARF10 is able to negatively regulate *JAG/FIL* and likely *AS* activity in the carpels and valves.

IV.1. New techniques to elucidate upstream regulators

We believe we have developed a protocol that may be successful in elucidating upstream regulatory genes. This series of experiments (Y1H, filtering using microarray data and TAT), when used together, can help establish new upstream regulators of many genes. Although the data generated from the use of any one single experiment alone may not be convincing, the combined impact of this approach appears to be very valuable.

Using the Y1H as a foundation we are able to visualize any possible connections between our promoter of interest and each transcription factor available in the TF library. This data is essentially useless unless it is filtered according to the process of interest. For example, we sorted our *MIR390* data using microarray data and continued our research only for TFs that appeared to be present in flowers and floral organs, but it is very possible to sort the Y1H candidate list using other process such as root, stem or leaf development. Next, an *in planta* experiment should be preformed to confirm the TF promoter recognition in a plant system. We used TAT, but other experiments such as protoplasts can be used. The results from this experiment can help in confirming the relationship between the TF and promoter of interest.

Overall, the results we obtained show that micoroRNAs play essential roles in fruit patterning and development. Although the genetic network controlling fruit

development already appears to be quite complex, we now realize the network is far from complete. Our data strongly suggests the addition of the miR156-SPL, miR390-TAS3-ARF3 and miR160-ARF10 regulatory nodes to this network. However, even with these additions, we believe there is still more work to be done in elucidating the upstream regulators of those miRNAs. Using the new technique just described, we strongly believe we can elucidate new upstream regulatory networks to help complete the genetic network controlling fruit development.

APPENDIX A: FIGURES AND FIGURE LEGENDS

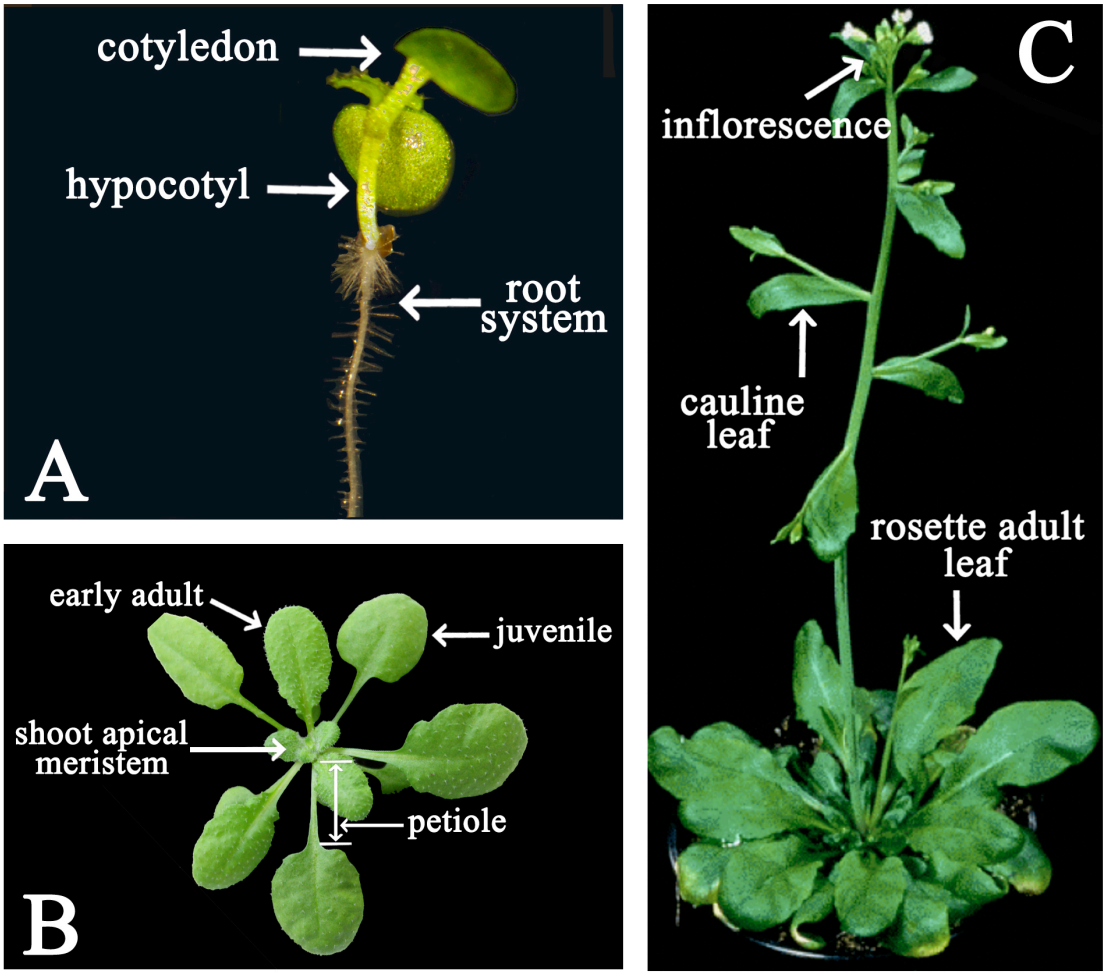


Figure 1. Vegetative Anatomy of *Arabidopsis thaliana.*
(**A**) Ten-day old seedling grown in continuous light with arrows indicating the location of the hypocotyl, cotyledons and root system. (**B**) Twenty-day old seedling grown in continuous light with arrows indicating the juvenile leaves, early adult leaves, petiole, and shoot apical meristem (SAM). (**C**) A fully reproductively competent adult plant bearing cauline leaves and an inflorescence.

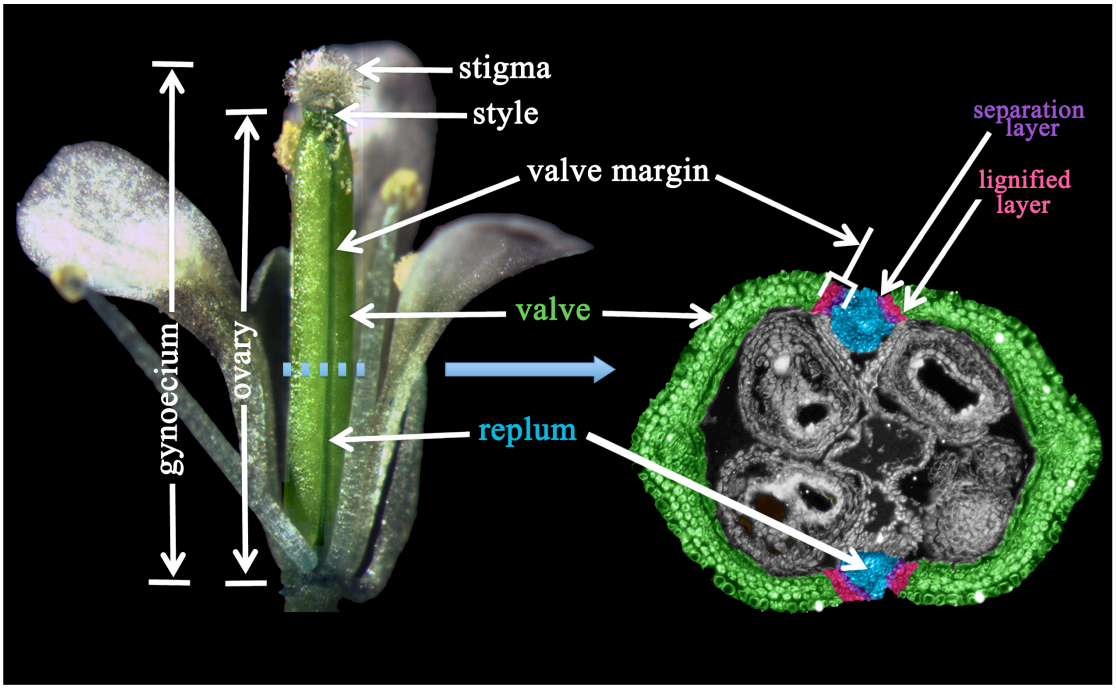


Figure 2. Reproductive Anatomy of *Arabidopsis thaliana.*
On the left, a whole mount picture of an adult flower bearing a stage 15 fruit. The main tissues of the *Arabidopsis* gynoecium have been indicated with arrows. Cross-section (right) of an *Arabidopsis* fruit at stage 16 in which the valves have been highlighted in green, replum in blue and valve margin in pink and purple. The valve margin is composed of two distinct layers; the separation layer (SL, highlighted in purple) and the lignified layer (LL, in pink).

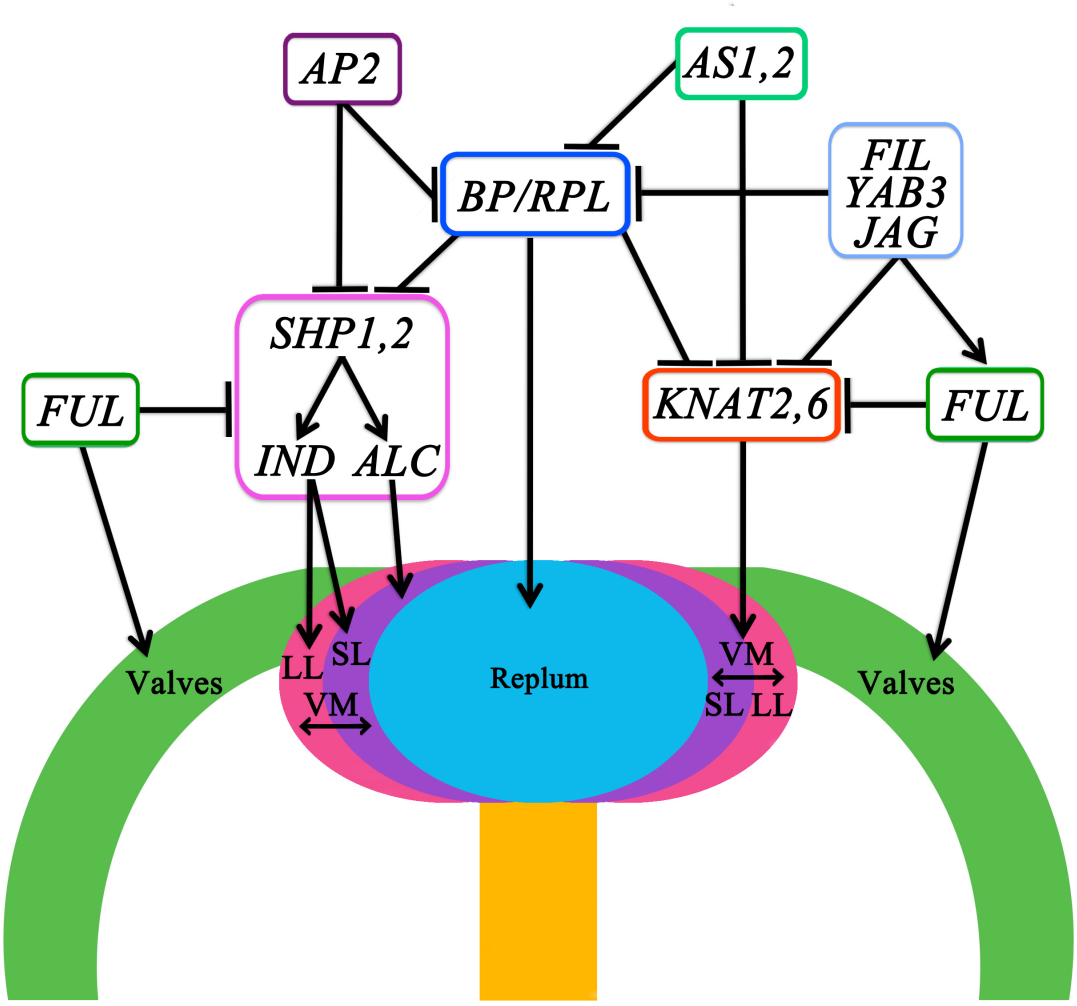
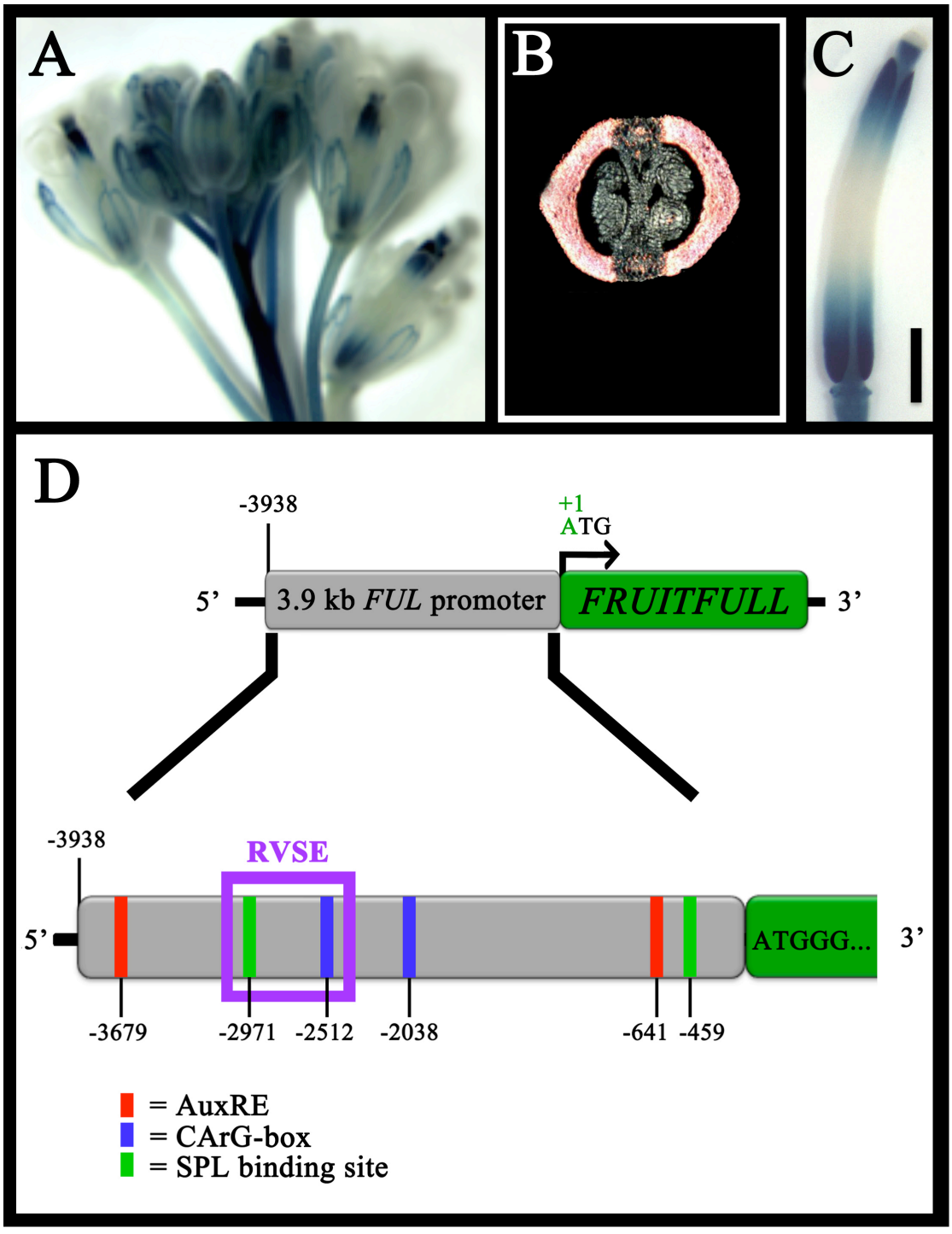


Figure 3. Current Genetic Network Controlling Fruit Patterning.
The model shows the current participating genes and the genetic interactions that take place during fruit (ovary) morphogenesis in *Arabidopsis.*

Figure 4. Expression Pattern of the Transgenic Reporter *FUL::GUS* in Reproductive Tissue.
(**A**) Whole mount staining of a *FUL::GUS* inflorescence. (**B**) Cross-section of a stage 13 *FUL::GUS* fruit in which the reporter signal is localized in the valve tissue. (**C**) Whole mount staining of a stage 16 fruit for *FUL::GUS* reporter lines showing the bipolar expression pattern of *FUL* in fruit valves. (**D**) Schematic representation of the *FUL* locus. By using *in silico* programs we identified six functional cis-regulatory motifs within the 3.9 kb 5' regulatory region (promoter) used for the *FUL::GUS* construct. We found two AuxRE motifs (red), two CArG-boxes (blue) and two SPL-binding sites (green). The purple square delimits the region of valve specific expression (RVSE). Scale bar: 1 mm.


A
B
C
D
-3938
+1
ATG
5'
3.9 kb FUL promoter
FRUITFULL
3'
-3938
RVSE
5'
ATGGG...
3'
-3679
-2971
-2512
-2038
-641
-459
= AuxRE
= CArG-box
= SPL binding site

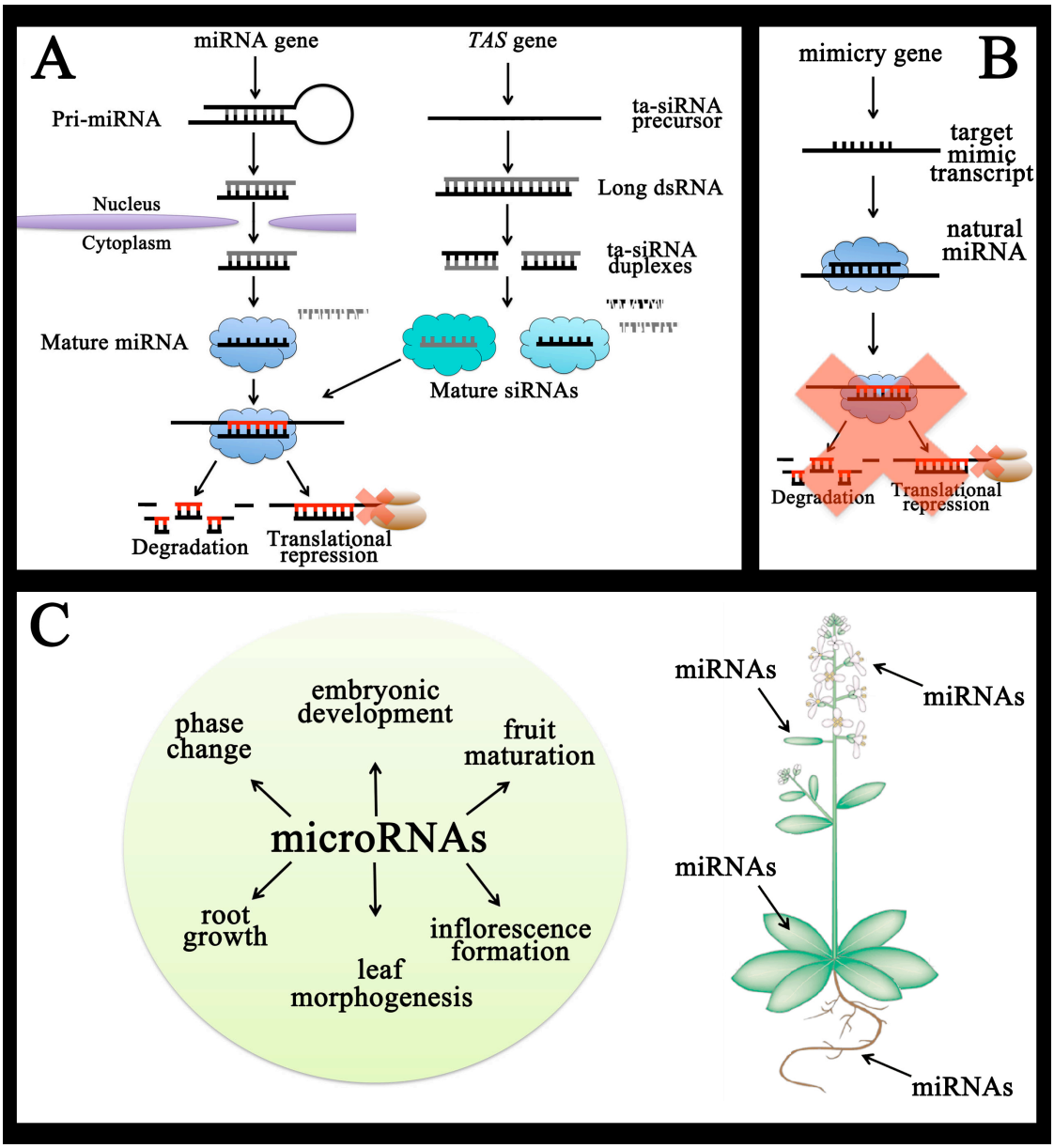


Figure 5. Biogenesis Pathways of MicroRNAs and Trans-Acting Small Interfering RNAs in *Arabidopsis.*

(A) Current model for generating functional microRNAs (miRNA) and trans-acting small interfering RNAs (ta-siRNA). (**B**) Model for miRNA inhibition by MIM (target mimicry) nucleic acid molecules. The repression of regulatory proteins by miRNAs and ta-siRNAs is an important control mechanism used to ensure proper timing and correct gene expression levels in the developing plant. (**C**) Developmental processes in which miRNAS and ta-siRNAs participate.

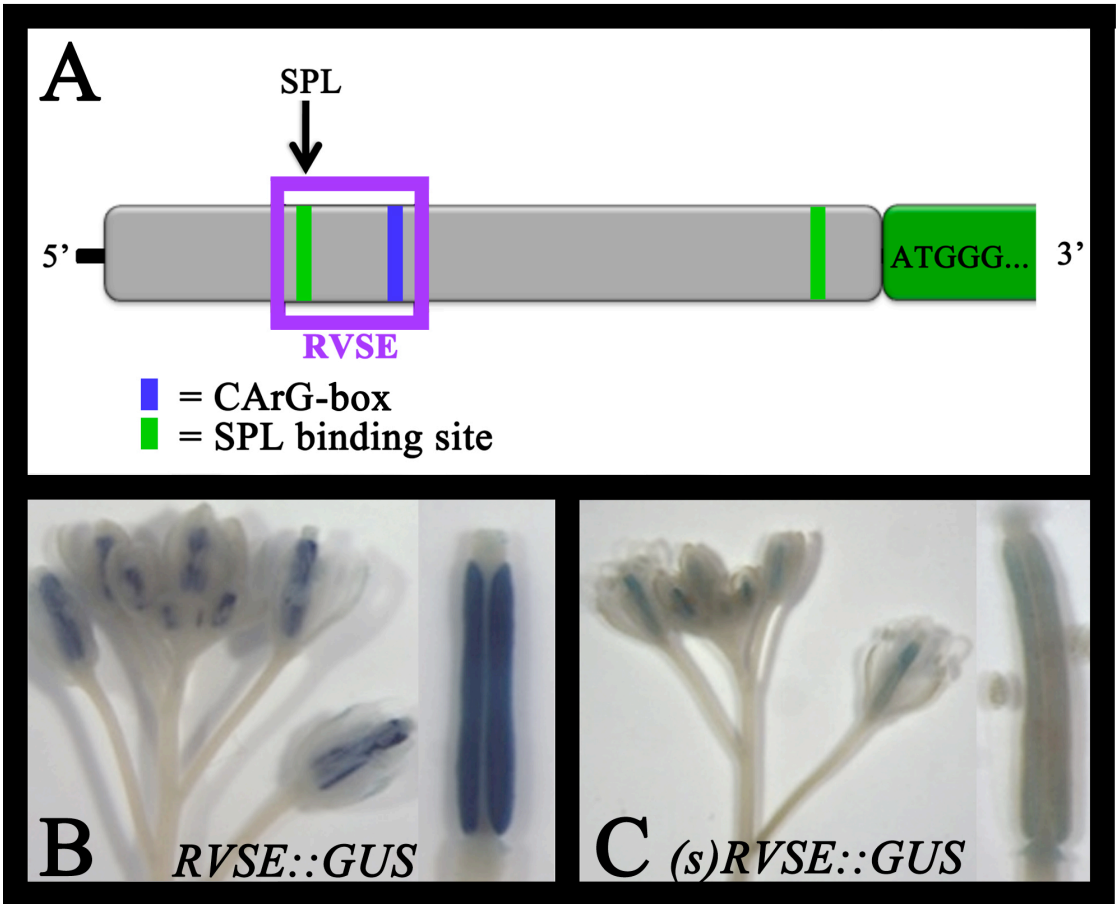


Figure 6. The miR156-SPL Regulatory Node Regulates *FUL* Activity in Fruit Valves.
(**A**) The RVSE *FUL* promoter fragment contains a SPL binding site (green) and a CArG-box motif (blue). (**B**) Whole mount staining of inflorescences (left) and stage 13 fruit (right) of *RVSE::GUS* transgenic plants. The *RVSE::GUS* reporters displays specific and uniform expression in carpels and valves. (**C**) Whole mount staining of inflorescences (left) and stage 15 fruit (right) of *(s)RVSE::GUS* transgenic plants. The *(s)RVSE::GUS* reporters lack the 5' SPL binding site. In these transgenic lines, the *GUS* activity in valves and carpels is drastically reduced when compared to that of *RVSE::GUS* plants.

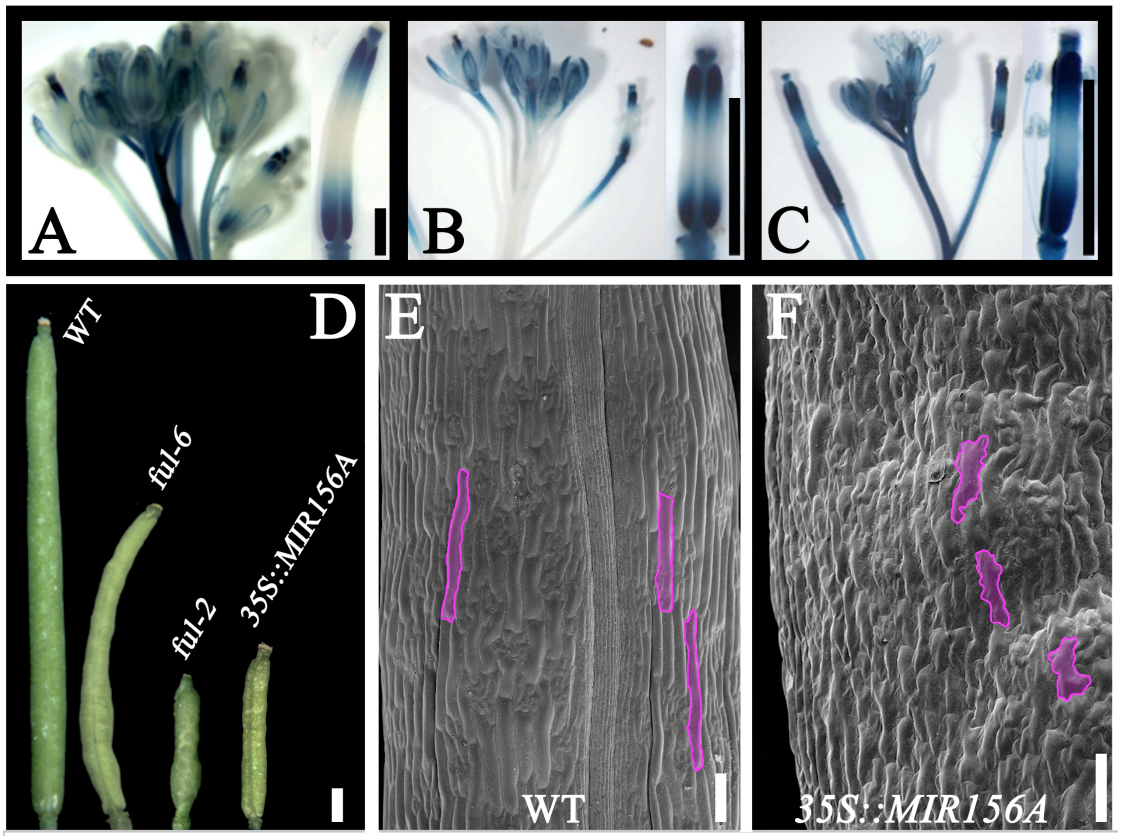
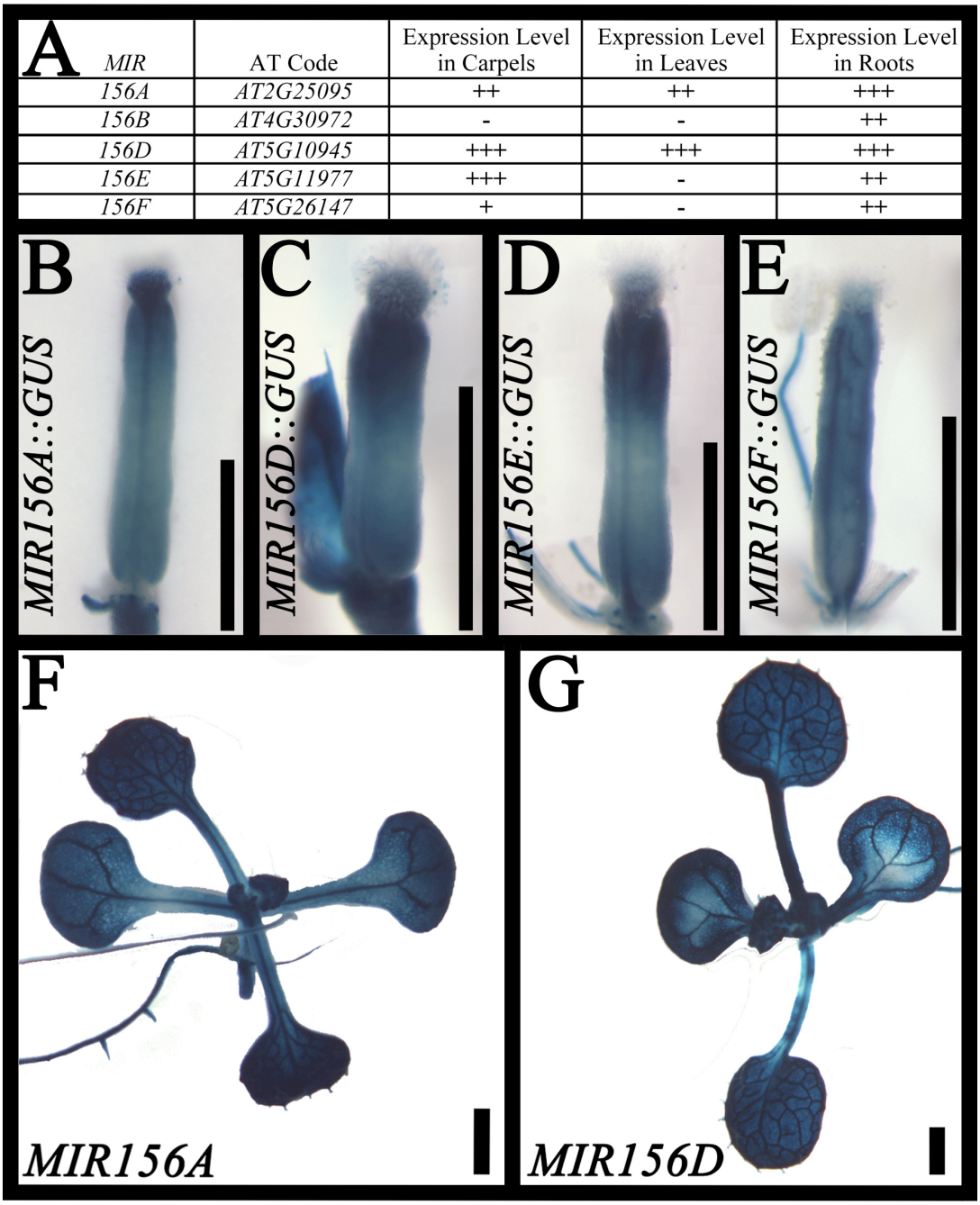


Figure 7. miR156 Indirectly Affects *FUL* Expression and Regulates the Size of Epidermal Cells in Fruit Valves.
Analysis of *FUL::GUS* activity in reproductive tissues of WT (**A**), *35S::SPL3*^{WT} (**B**) and *35S::SPL3*^{miR156R} (**C**) backgrounds. **(D)** Wild-type (WT), *ful-6* (weak mutant allele), *ful-2* (strong allele) and *35S::MIR156A* stage 17 fruits. SEM micrographs of WT (**E**) and *35S::MIR156A* (**F**) fruits in which some epidermal cells have been shaded in purple. Scale bars; 1mm in A, D, E, F; 100 μm in B,C. Scale bars apply to fruits only.



MIR	AT Code	Expression Level in Carpels	Expression Level in Leaves	Expression Level in Roots
156A	*AT2G25095*	++	++	+++
156B	*AT4G30972*	-	-	++
156D	*AT5G10945*	+++	+++	+++
156E	*AT5G11977*	+++	-	++
156F	*AT5G26147*	+	-	++

Figure 8. Expression Patterns of *MIR156::GUS* Reporters.
(**A**) Table containing the AT code for each *MIR156* gene and indication of where expression is found for each transgenic line. Three "+" indicate high levels of expression, two indicate medium levels of expression, one indicates low levels of expression and "–" indicates no expression. Whole mount *GUS* staining of stage 14 fruits for (**B**) *MIR156A* reporter, (**C**) *MIR156D* reporter, (**D**) *MIR156E* reporter, and (**E**) *MIR156F* reporter. Whole mount staining of seedlings 12 days after germination for reporter lines (**F**) *MIR156A*, (**G**) *MIR156D*. All scale bars to 1 mm.

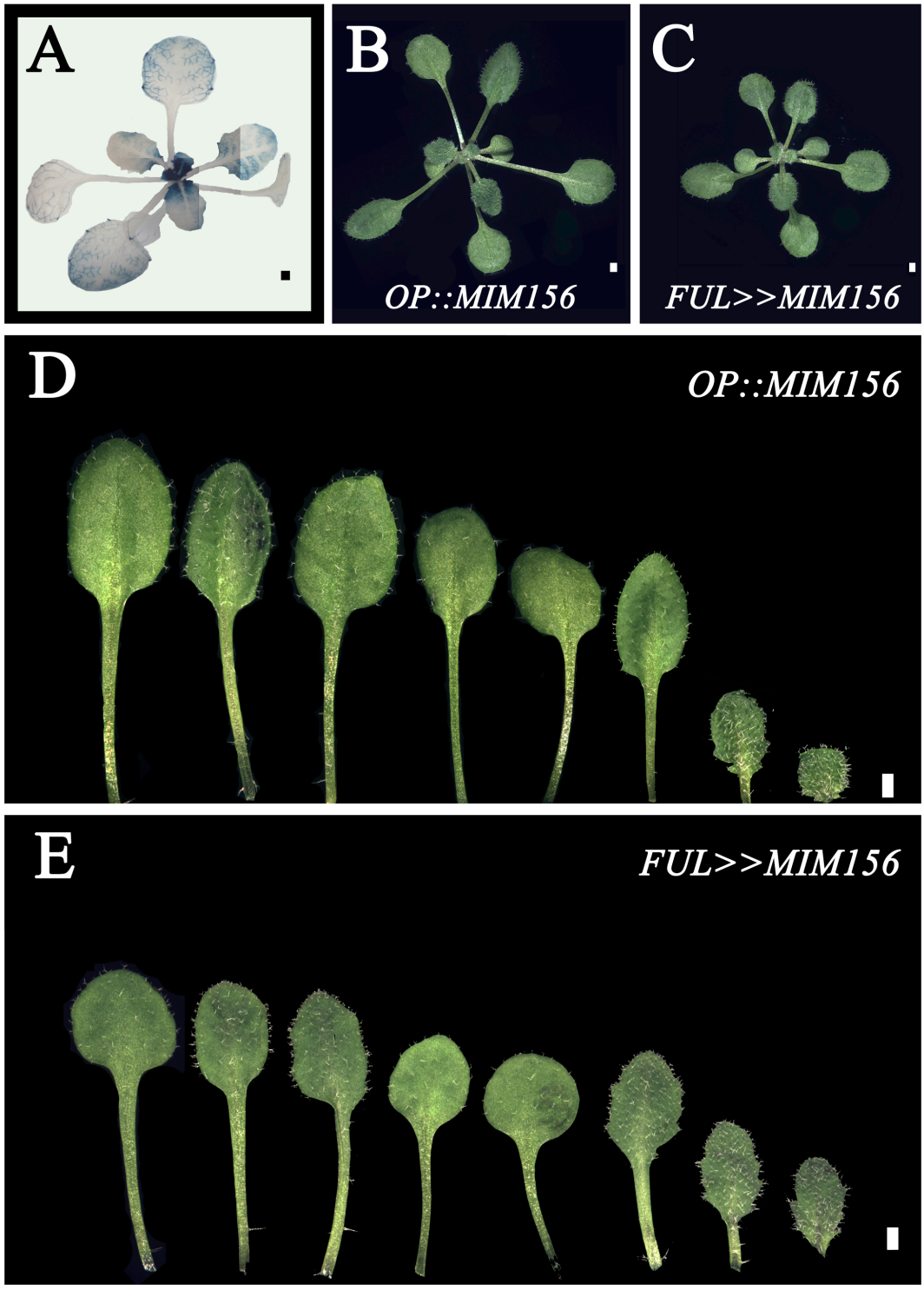


Figure 9. Leaf Phenotype of *FUL>>MIM156* Plants.
(**A**) Whole mount *GUS* staining of a 20 day old *FUL::GUS* seedling. Leaf morphology in *FUL>>MIM156* plants (**C-E**). *OP::MIM156* plants were used as a control (**B-D**). All scale bars to 1 mm.

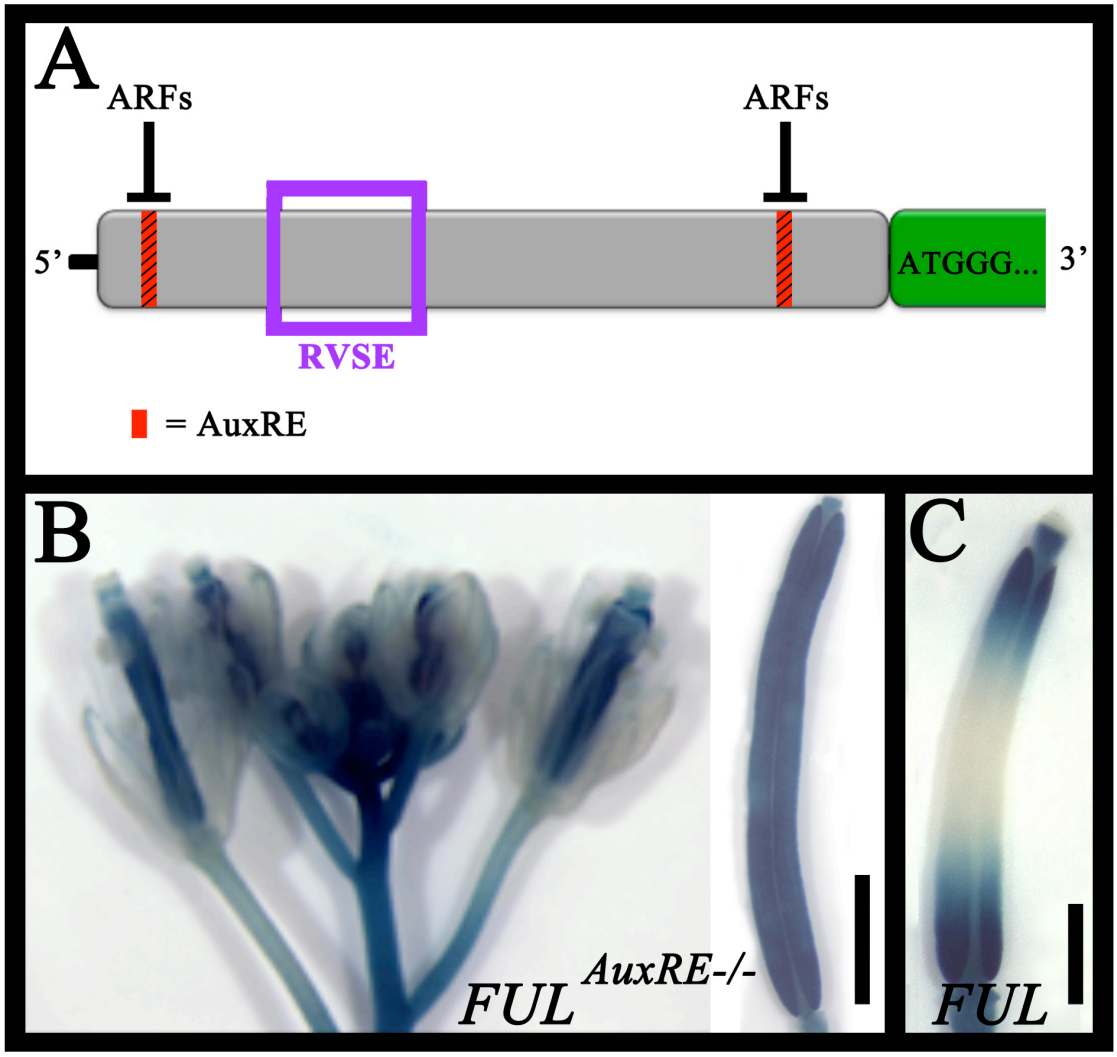


Figure 10. AuxRe cis-Regulatory Motifs are Involved in Promoting the Bipolar Expression pattern of the *FUL* gene in the Valves.
(A) Diagram of the *FUL* promoter with the location of the two AuxREs (red). (**B**) Whole mount staining of $FUL^{AuxRE-/-}$*::GUS* inflorescence (left) and stage 17 fruit (right). Mutation of both AuxREs (black dashes) causes abolition of the typical bipolar expression pattern that *FUL::GUS* adopts after fertilization in the valves (**C**). Scale bars are 1 mm and apply only to fruits.

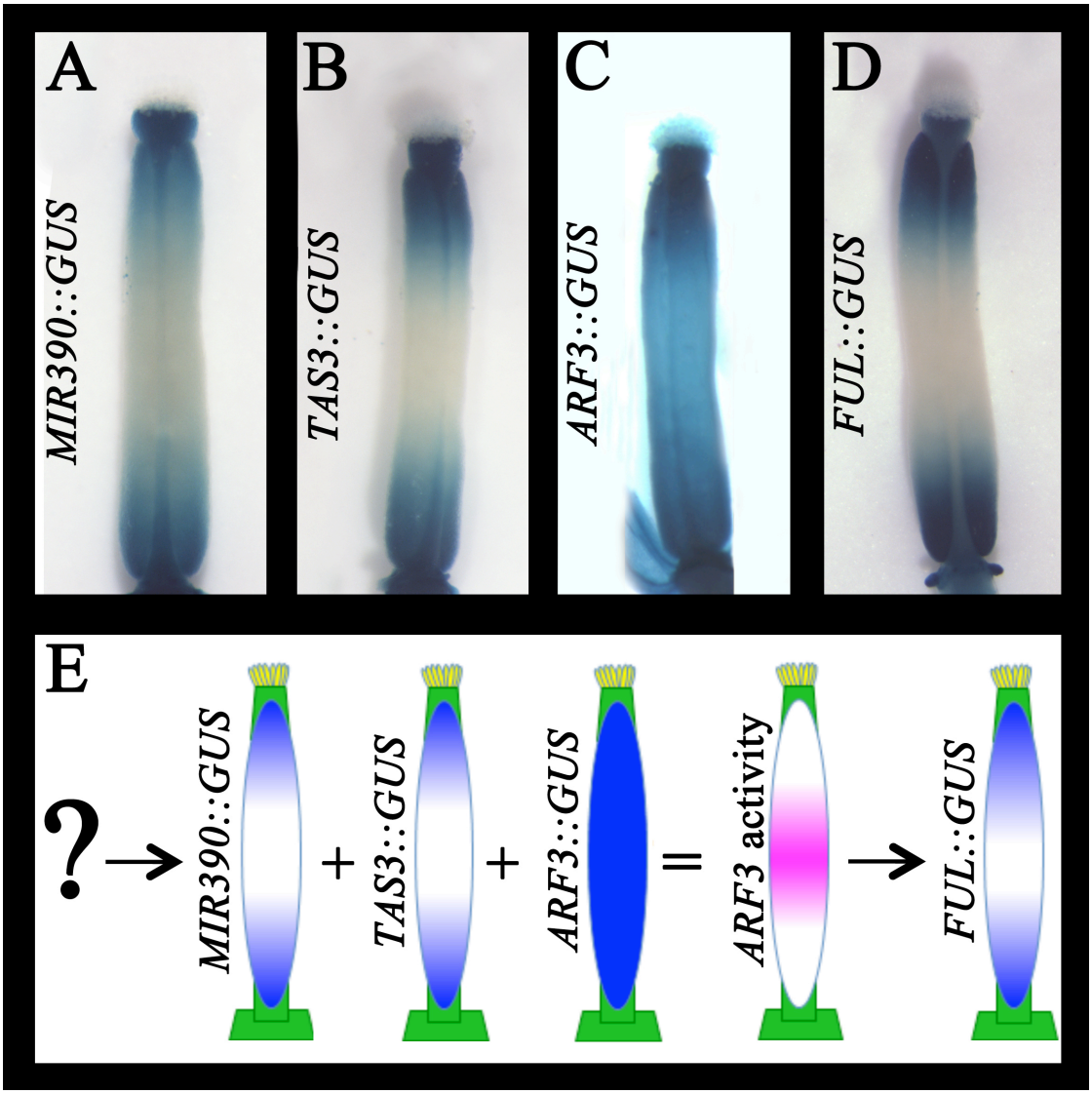


Figure 11. The miR390/*TAS3-ARF3* Module is likely Involved in Promoting the Bipolar Expression Pattern of *FUL* in Valve Tissue.
(**A-D**) Whole mount staining of stage 15 fruits for **(A)** *MIR390::GUS* reporter, **(B)** *TAS3::GUS* reporter, **(C)** *ARF3::GUS* reporter, and **(D)** *FUL::GUS* reporter. **(E)** Working model for how activities of *miR390* and *TAS3* work to restrict *ARF3* to the middle region of the valve, where it likely represses *FUL* expression. All fruits are the same scale.

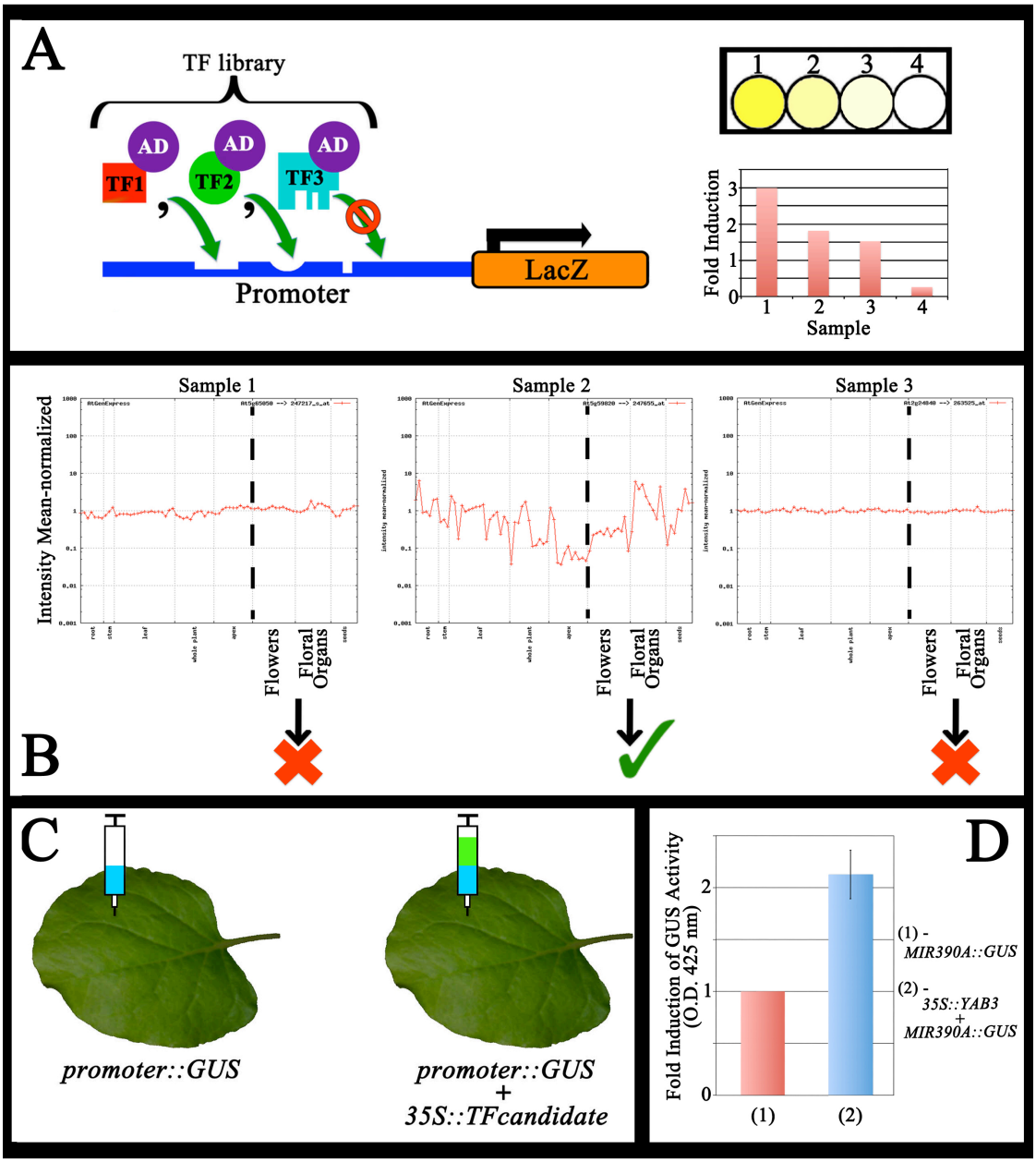


Figure 12. Identification of Upstream Regulators of *MIR390*.
(**A**) Schematic representation of the Y1H approach used to identify upstream regulators of *MIR390*. (**B**) Transcriptome analysis of the putative up-stream candidates for *MIR390* using the AtGenExpress web-based tool. Further experiments were done with the candidates highly expresses in reproductive tissues. (**C**) Transient tobacco assay to test TF-promoter interaction *in planta*. (**D**) *in vitro GUS* assay for one of the upstream candidates (YAB3) identified for *MIR390*.

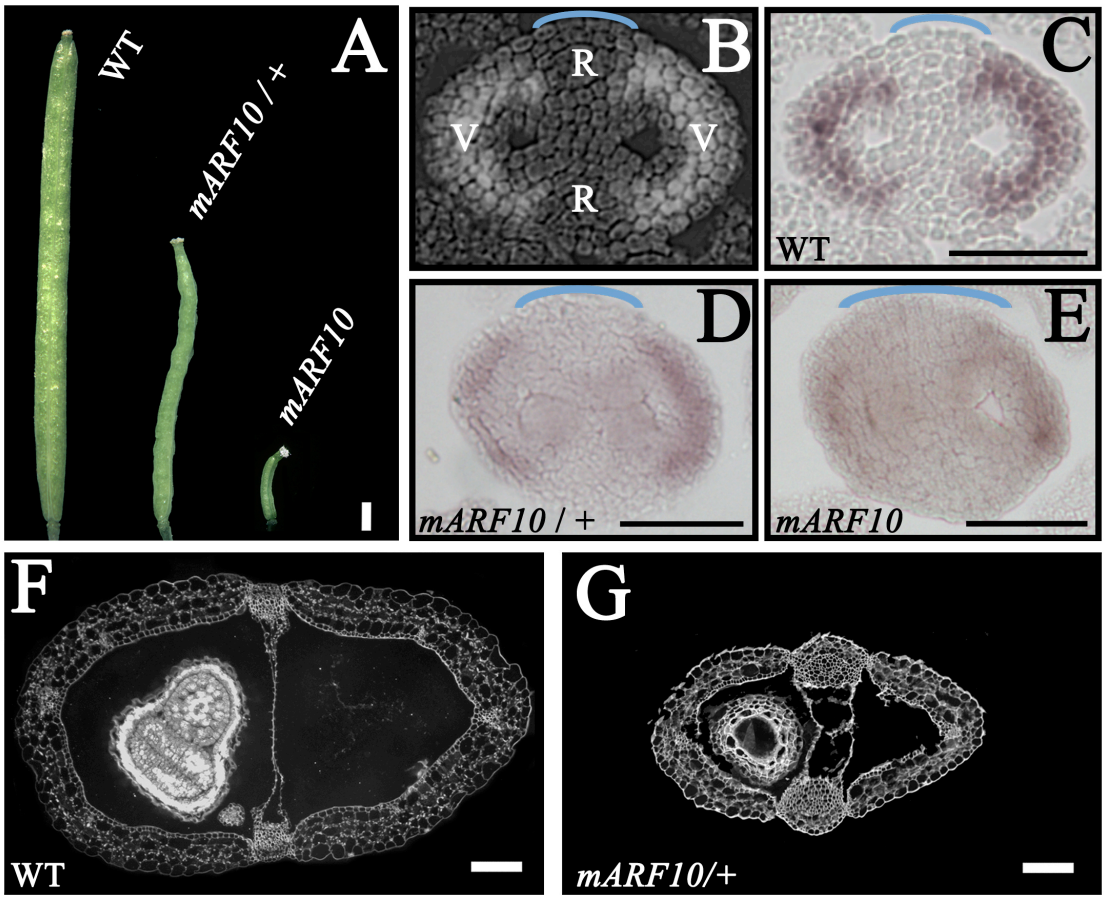


Figure 13. Reproductive Phenotypes of Transgenic Plants Misexpressing a miR160-Resistant Version of *ARF10 (mARF10).*
(A) From left to right: fruits at stage 17 from wild-type, hemizygous miR160-resistant *ARF10* (*mARF10/+*) and homozygous miR160-resistant *ARF10* (*mARF10*) plants. (**B**) Cross-section of an *Arabidopsis* gynoecium at stage 8. The carpel-valves (V) have been artificially colored in light grey. The size of the medial region, from which the replum (R in grey) will arise, is delimited with a blue arc. Expression of *FIL* detected by mRNA *in situ* hybridization using an anti-sense probe in stage 8 WT (**C**), stage 9 *mARF10/+* (**D**) and stage 9 *mARF10* (**E**) gynoecium. (**F-G**) Cross-sections of stage 17 fruits revealed a decrease in valve size and increase in replum size in *mARF10/+* (**G**) when compared to wild-type (**F**). Scale bars; 50 µm in C,D,E; 100 µm in F,G.

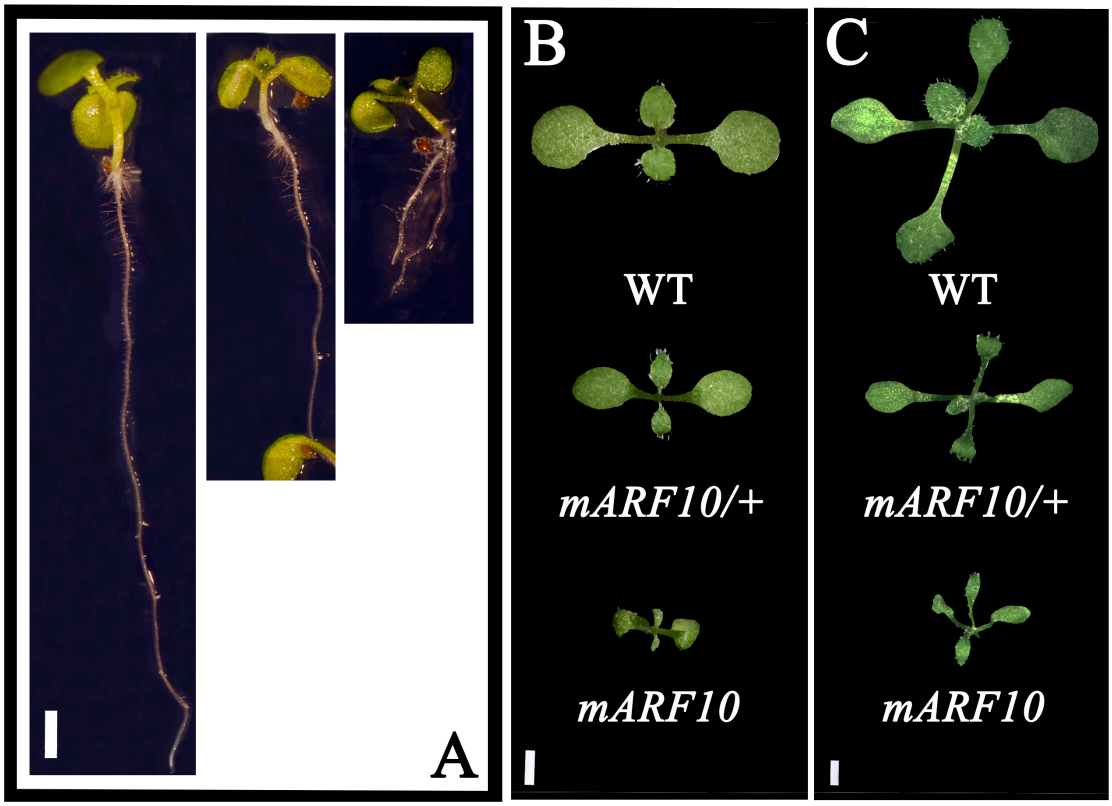


Figure 14. Misexpression of *mARF10* Affects Vegetative Development.
(**A**) Root phenotype of wild-type (left), *mARF10/+* (middle) and *mARF10* (right) seedlings. (**B**) 8 day old seedlings of wild-type (top), *mARF10/+* (middle) and *mARF10* (bottom). (**C**) 12 day old seedlings of wild-type (top), *mARF10/+* (middle) and *mARF10* (bottom). All scale bars are 1 mm.

APPENDIX B: TABLES AND TABLE LEGENDS

Table 1. Oliogonucleotides used to create *GUS* reporter constructs.
The oligonucleotides (primers) listed were used to amplify the putative 5' promoter regions of the *MIR156* genes and create restriction sites for cloning into the vector pJJGUS for generating *GUS* reporter lines. Underlined are the restriction sites used for cloning.

R.E. = Restriction Enzyme

Gene	Length (bp)	5' Primer (5'-3')	3' Primer (5'-3')	5' R.E.	3' R.E.
MIR156A (AT2G25095)	2495	oJJR255 TTGGTACCACATTAT-TGGAGAAGATGCAAGC	oJJR256 TTGTCGACGTTTCT-TTGCGTTTCTCTTGTC	KpnI	SalI
MIR156B (AT4G30972)	2577	oJJR257 TTGTCGACGTTTCT-TTGCGTTTCTCTTGTC	oJJR258 TTCTCGAGGTTTTCTC-TGTTGCATTCCTCAATC	KpnI	XhoI
MIR156D (AT5G10945)	2154	oJJR261 TTGGTACCACTTCT-TTTCCCCCATCAAC	oJJR262 TTGTCGACGCTTCAAG-GTAAAGCATCAGAGC	KpnI	SalI
MIR156E (AT5G11977)	2547	oJJR263 TTGGTACCGCATATTCG-TTCCCACCACGTGTC	oJJR264 TTGTCGACCCTCCTA-ATTACCTTTCACACTC	KpnI	SalI
MIR156F (AT5G26147)	2783	oJJR266 TTGGTACCCAGGCT-GGGCATTCTCCACATGC	oJJR267 TTGGGCCCCCATCAAT-TCCTCACCACTC	KpnI	ApaI

Table 2. Oliogonucleotides used to amplify *MIM156* construct.
The oligonucleotides (primers) listed were used to amplify the MIM156 from the pGemT vector and clone into pJB36 10xOP.

Length (bp)	5' Primer (5'-3')	3' Primer (5'-3')	5' R.E.	3' R.E.
~500	oJJR267 TTGGTACCAAACACCACAAAA ACAAAAGAAAAATGGCCATC	oJJR268 TTGGATCCAAGAGGAATTC ACTATAAAGAGAATCGG	KpnI	BamHI

Table 3. Oliogonucleotides used to create *pLacZi* constructs.
The oligonucleotides (primers) listed were used to amplify fragments of the putative 5' promoter region of *MIR390* gene (AT2G38325) and create restriction sites for cloning into the binary vector pLacZi. Underlined are the restriction sites used for cloning.

R.E.= Restriction Enzyme

MIR390 fragment	Length (bp)	5' Primer (5'-3')	3' Primer (5'-3')	5' R.E.	3' R.E.
MIR390-1	710	oLJB1 TTGGTACCCTGACCGGT-AAATTGGCAATAGAC	oLJB2 TTGTCGACCATCTCCA-TATCTTTTACTGGCTG	KpnI	SalI
MIR390-2	668	oLJB3 TTGGTACCCAGCCAGTA-AAAGATATGGAGATG	oLJB4 TTGTCGACGCTGGTAA-GTTTTGATTGCAATTG	KpnI	SalI
MIR390-3	706	oLJB5 TTGGTACCCAATTGCAA-TCAAAACTTACCAGC	oLJB6 TTGTCGACCCTGCCGA-CTACGGTACTAGTC	KpnI	SalI
MIR390-4	378	oLJB7 TTGGTACCGACTAGTAC-CGTAGTCGGCAGG	oLJB8 TTGTCGACGACCAATGC-TTATTTGCAAACTATGAG	KpnI	SalI
MIR390-5	633	oLJB9 TTGGTACCCTCATAGTT-TGCAAATAAGCATTGGTC	oLJB10 TTGTCGACTTGGGTTG-TGACTTAGAGAAAG	KpnI	SalI

Table 4. Transient assay in tobacco *GUS* expression for *MIR390::GUS* and *35S::YAB3* constructs

The OD_{420} of agrobacterium-infiltrated leaves taken after protein extraction. Fold induction for each was normalized to the control *MIR390::GUS*.

Construct	Experiment 1A	Experiment 2A	Experiment 1B	Experiment 2B
35S::YAB3	0.018	0.02	0.01	0.015
MIR390::GUS	1.30	2.80	2.60	2.20
MIR390::GUS + 35S::YAB3	3.20	3.50	5.50	4.38
Fold induction	2.46	1.94	2.11	1.99

REFERENCES

Allen, E., Xie, Z., Gustafson, A.M., Carrington, J.C., 2005. microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* 121, 207-221.

Alonso-Cantabrana, H., Ripoll, J.J., Ochando, I., Vera, A., Ferrándiz, C., Martínez-Laborda, A., 2007. Common regulatory networks in leaf and fruit patterning revealed by mutations in the Arabidopsis ASYMMETRIC LEAVES1 gene. *Development* 134, 2663-2671.

Arabidopsis Genome, I., 2000. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature* 408, 796-815.

Araki, T., 2001. Transition from vegetative to reproductive phase. *Current Opinion in Plant Biology* 4, 63-68.

Aukerman, M.J., Sakai, H., 2003. Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. *Plant Cell* 15, 2730-2741.

Axtell, M.J., Jan, C., Rajagopalan, R., Bartel, D.P., 2006. A two-hit trigger for siRNA biogenesis in plants. *Cell* 127, 565-577.

Baroux, C., Blanvillain, R., Betts, H., Batoko, H., Craft, J., Martinez, A., Gallois, P., Moore, I., 2005. Predictable activation of tissue-specific expression from a single gene locus using the pOp/LhG4 transactivation system in Arabidopsis. *Plant Biotechnol J* 3, 91-101.

Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281-297.

Bhatt, A.M., Etchells, J.P., Canales, C., Lagodienko, A., Dickinson, H., 2004. VAAMANA--a BEL1-like homeodomain protein, interacts with KNOX proteins BP and STM and regulates inflorescence stem growth in Arabidopsis. *Gene* 328, 103-111.

Bowman, J., 1993. Arabidopsis An Atlas of Morphology and Development. Springer-Verlag.

Brand, L., Horler, M., Nuesch, E., Vassalli, S., Barrell, P., Yang, W., Jefferson, R.A., Grossniklaus, U., Curtis, M.D., 2006. A versatile and reliable two-component system for tissue-specific gene induction in Arabidopsis. *Plant physiology* 141, 1194-1204.

Byrne, M.E., Barley, R., Curtis, M., Arroyo, J.M., Dunham, M., Hudson, A., Martienssen, R.A., 2000. Asymmetric leaves1 mediates leaf patterning and stem cell function in Arabidopsis. *Nature* 408, 967-971.

Byrne, M.E., Groover, A.T., Fontana, J.R., Martienssen, R.A., 2003. Phyllotactic pattern and stem cell fate are determined by the Arabidopsis homeobox gene BELLRINGER. *Development* 130, 3941-3950.

Byrne, M.E., Simorowski, J., Martienssen, R.A., 2002. ASYMMETRIC LEAVES1 reveals knox gene redundancy in Arabidopsis. *Development* 129, 1957-1965.

Cardon, G., Hohmann, S., Klein, J., Nettesheim, K., Saedler, H., Huijser, P., 1999. Molecular characterisation of the Arabidopsis SBP-box genes. *Gene* 237, 91-104.

Cardon, G.H., Hohmann, S., Nettesheim, K., Saedler, H., Huijser, P., 1997. Functional analysis of the Arabidopsis thaliana SBP-box gene SPL3: a novel gene involved in the floral transition. *The Plant journal : for cell and molecular biology* 12, 367-377.

Carrington, J.C., Ambros, V., 2003. Role of microRNAs in plant and animal development. *Science* 301, 336-338.

Chapman, E.J., Estelle, M., 2009. Mechanism of auxin-regulated gene expression in plants. *Annu Rev Genet* 43, 265-285.

Chen, X., 2005. MicroRNA biogenesis and function in plants. FEBS Lett 579, 5923-5931.

Chitwood, D.H., Nogueira, F.T., Howell, M.D., Montgomery, T.A., Carrington, J.C., Timmermans, M.C., 2009. Pattern formation via small RNA mobility. *Genes Dev* 23, 549-554.

Chitwood, D.H., Timmermans, M.C., 2010. Small RNAs are on the move. *Nature* 467, 415-419.

Clough, S.J., Bent, A.F., 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* 16, 735-743.

Deplancke, B., Dupuy, D., Vidal, M., Walhout, A.J., 2004. A gateway-compatible yeast one-hybrid system. *Genome research* 14, 2093-2101.

Dinneny, J.R., Weigel, D., Yanofsky, M.F., 2005. A genetic framework for fruit patterning in Arabidopsis thaliana. *Development* 132, 4687-4696.

Dinneny, J.R., Weigel, D., Yanofsky, M.F., 2006. NUBBIN and JAGGED define stamen and carpel shape in Arabidopsis. *Development* 133, 1645-1655.

Dinneny, J.R., Yadegari, R., Fischer, R.L., Yanofsky, M.F., Weigel, D., 2004. The role of JAGGED in shaping lateral organs. *Development* 131, 1101-1110.

Fahlgren, N., Montgomery, T.A., Howell, M.D., Allen, E., Dvorak, S.K., Alexander, A.L., Carrington, J.C., 2006. Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in Arabidopsis. *Curr Biol* 16, 939-944.

Felippes, F.F., Ott, F., Weigel, D., 2010. Comparative analysis of non-autonomous effects of tasiRNAs and miRNAs in Arabidopsis thaliana. *Nucleic Acids Res*.

Felippes, F.F., Weigel, D., 2009. Triggering the formation of tasiRNAs in Arabidopsis thaliana: the role of microRNA miR173. *EMBO Rep* 10, 264-270.

Fernandez, A.I., Viron, N., Alhagdow, M., Karimi, M., Jones, M., Amsellem, Z., Sicard, A., Czerednik, A., Angenent, G., Grierson, D., May, S., Seymour, G., Eshed, Y., Lemaire-Chamley, M., Rothan, C., Hilson, P., 2009. Flexible tools for gene expression and silencing in tomato. *Plant physiology* 151, 1729-1740.

Ferrándiz, C., 2002. Regulation of fruit dehiscence in Arabidopsis. *J Exp Bot* 53, 2031-2038.

Ferrándiz, C., Gu, Q., Martienssen, R., Yanofsky, M.F., 2000a. Redundant regulation of meristem identity and plant architecture by FRUITFULL, APETALA1 and CAULIFLOWER. *Development* 127, 725-734.

Ferrándiz, C., Liljegren, S.J., Yanofsky, M.F., 2000b. Negative regulation of the SHATTERPROOF genes by FRUITFULL during Arabidopsis fruit development. *Science* 289, 436-438.

Ferrándiz, C., Pelaz, S., Yanofsky, M.F., 1999. Control of carpel and fruit development in Arabidopsis. Annu Rev Biochem 68, 321-354.
Fornara, F., Coupland, G., 2009. Plant Phase Transitions Make a SPLash. *Cell* 138, 625-627.

Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., Garcia, J.A., Paz-Ares, J., 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat Genet* 39, 1033-1037.

Gandikota, M., Birkenbihl, R.P., Hohmann, S., Cardon, G.H., Saedler, H., Huijser, P., 2007. The miRNA156/157 recognition element in the 3' UTR of the Arabidopsis SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings. *Plant J* 49, 683-693.

Girin, T., Sorefan, K., Ostergaard, L., 2009. Meristematic sculpting in fruit development. *J Exp Bot* 60, 1493-1502.

Gu, Q., Ferrándiz, C., Yanofsky, M.F., Martienssen, R., 1998. The FRUITFULL MADS-box gene mediates cell differentiation during Arabidopsis fruit development. *Development* 125, 1509-1517.

Guo, M., Thomas, J., Collins, G., Timmermans, M.C., 2008. Direct repression of KNOX loci by the ASYMMETRIC LEAVES1 complex of Arabidopsis. *Plant Cell* 20, 48-58.

Hellens, R., Mullineaux, P., Klee, H., 2000. Technical Focus:a guide to Agrobacterium binary Ti vectors. *Trends in plant science* 5, 446-451.

Huijser, P., Schmid, M., 2011. The control of developmental phase transitions in plants. *Development* 138, 4117-4129.

Hunter, C., Willmann, M.R., Wu, G., Yoshikawa, M., de la Luz Gutierrez-Nava, M., Poethig, S.R., 2006. Trans-acting siRNA-mediated repression of ETTIN and ARF4 regulates heteroblasty in Arabidopsis. *Development* 133, 2973-2981.

Janosevic, D., Uzelac, B., Stojicic, D., Budimir, S., 2007. Developmental anatomy of cotyledons and leaves in has mutant of Arabidopsis thaliana. *Protoplasma* 231, 7-13.

Jiao, Y., Wang, Y., Xue, D., Wang, J., Yan, M., Liu, G., Dong, G., Zeng, D., Lu, Z., Zhu, X., Qian, Q., Li, J., 2010. Regulation of OsSPL14 by OsmiR156 defines ideal plant architecture in rice. *Nature genetics* 42, 541-544.

Kim, J.J., Lee, J.H., Kim, W., Jung, H.S., Huijser, P., Ahn, J.H., 2012. The microRNA156-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3 Module Regulates Ambient Temperature-Responsive Flowering via FLOWERING LOCUS T in Arabidopsis. *Plant physiology* 159, 461-478.

Kim, J.Y., Yuan, Z., Jackson, D., 2003. Developmental regulation and significance of KNOX protein trafficking in Arabidopsis. *Development* 130, 4351-4362.

Kumaran, M.K., Bowman, J.L., Sundaresan, V., 2002. YABBY polarity genes mediate the repression of KNOX homeobox genes in Arabidopsis. *Plant Cell* 14, 2761-2770.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L., Yanofsky, M.F., 2000. SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. *Nature* 404, 766-770.

Liljegren, S.J., Roeder, A.H., Kempin, S.A., Gremski, K., Ostergaard, L., Guimil, S., Reyes, D.K., Yanofsky, M.F., 2004. Control of fruit patterning in Arabidopsis by INDEHISCENT. *Cell* 116, 843-853.

Liu, P.P., Montgomery, T.A., Fahlgren, N., Kasschau, K.D., Nonogaki, H., Carrington, J.C., 2007. Repression of AUXIN RESPONSE FACTOR10 by microRNA160 is critical for seed germination and post-germination stages. *Plant J* 52, 133-146.

Liu, Z., Jia, L., Wang, H., He, Y., 2011. HYL1 regulates the balance between adaxial and abaxial identity for leaf flattening via miRNA-mediated pathways. *Journal of experimental botany* 62, 4367-4381.

Mai, Q.-A.N., 2009. Uncovering new regulators of Arabidopsis thaliana fruit morphogenesis. Ms Thesis.

Mallory, A.C., Bartel, D.P., Bartel, B., 2005. MicroRNA-directed regulation of Arabidopsis AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. *Plant Cell* 17, 1360-1375.

Marin, E., Jouannet, V., Herz, A., Lokerse, A.S., Weijers, D., Vaucheret, H., Nussaume, L., Crespi, M.D., Maizel, A., 2010. miR390, Arabidopsis TAS3 tasiRNAs, and their AUXIN RESPONSE FACTOR targets define an autoregulatory network quantitatively regulating lateral root growth. *Plant Cell* 22, 1104-1117.

Melnyk, C.W., Molnar, A., Baulcombe, D.C., 2011. Intercellular and systemic movement of RNA silencing signals. *The EMBO journal* 30, 3553-3563.

Melzer, S., Lens, F., Gennen, J., Vanneste, S., Rohde, A., Beeckman, T., 2008. Flowering-time genes modulate meristem determinacy and growth form in Arabidopsis thaliana. *Nature genetics* 40, 1489-1492.

Meyerowitz, E.M., 1989. Arabidopsis, a useful weed. Cell 56, 263-269.
Miura, K., Ikeda, M., Matsubara, A., Song, X.J., Ito, M., Asano, K., Matsuoka, M., Kitano, H., Ashikari, M., 2010. OsSPL14 promotes panicle branching and higher grain productivity in rice. *Nature genetics* 42, 545-549.

Murai, K., Miyamae, M., Kato, H., Takumi, S., Ogihara, Y., 2003. WAP1, a wheat APETALA1 homolog, plays a central role in the phase transition from vegetative to reproductive growth. *Plant & cell physiology* 44, 1255-1265.

Nguyen, A.L., 2007. Transcriptional regulation of FRUITFULL, a MADS-box gene involved in Arabidopsis fruit development. Ms Thesis.

Ohno, C.K., Reddy, G.V., Heisler, M.G., Meyerowitz, E.M., 2004. The Arabidopsis JAGGED gene encodes a zinc finger protein that promotes leaf tissue development. *Development* 131, 1111-1122.

Ori, N., Eshed, Y., Chuck, G., Bowman, J.L., Hake, S., 2000. Mechanisms that control knox gene expression in the Arabidopsis shoot. Development 127, 5523-5532.
Park, M.Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H., Poethig, R.S., 2005. Nuclear processing and export of microRNAs in Arabidopsis. *Proc Natl Acad Sci U S A* 102, 3691-3696.

Poethig, R.S., 2009. Small RNAs and developmental timing in plants. *Curr Opin Genet Dev* 19, 374-378.

Pruneda-Paz, J.L., Breton, G., Para, A., Kay, S.A., 2009. A functional genomics approach reveals CHE as a component of the Arabidopsis circadian clock. *Science* 323, 1481-1485.

Ragni, L., Belles-Boix, E., Gunl, M., Pautot, V., 2008. Interaction of KNAT6 and KNAT2 with BREVIPEDICELLUS and PENNYWISE in Arabidopsis inflorescences. *Plant Cell* 20, 888-900.

Rajani, S., Sundaresan, V., 2001. The Arabidopsis myc/bHLH gene ALCATRAZ enables cell separation in fruit dehiscence. *Curr Biol* 11, 1914-1922.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., Bartel, D.P., 2002. Prediction of plant microRNA targets. *Cell* 110, 513-520.

Ripoll, J.J., Ferrándiz, C., Martínez-Laborda, A., Vera, A., 2006. PEPPER, a novel K-homology domain gene, regulates vegetative and gynoecium development in Arabidopsis. *Dev Biol* 289, 346-359.

Ripoll, J.J., Roeder, A.H., Ditta, G.S., Yanofsky, M.F., 2011. A novel role for the floral homeotic gene APETALA2 during Arabidopsis fruit development. *Development* 138, 5167-5176.

Robles, P., Pelaz, S., 2005. Flower and fruit development in Arabidopsis thaliana. *The International journal of developmental biology* 49, 633-643.

Roeder, A.H., Ferrandiz, C., Yanofsky, M.F., 2003. The role of the REPLUMLESS homeodomain protein in patterning the Arabidopsis fruit. *Curr Biol* 13, 1630-1635.

Roeder, A.H., Yanofsky, M.F., 2006. Fruit development in Aabidopsis, in: CR Somerville, E.M. (Ed.), The Arabidopsis Book. American Society of Plant Biologists, Rockville, MD, doi: 10.1199/tab.0074, http://www.aspb.org/publications/arabidopsis/.

Sawa, S., Watanabe, K., Goto, K., Liu, Y.G., Shibata, D., Kanaya, E., Morita, E.H., Okada, K., 1999. FILAMENTOUS FLOWER, a meristem and organ identity gene of Arabidopsis, encodes a protein with a zinc finger and HMG-related domains. *Genes & development* 13, 1079-1088.

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Scholkopf, B., Weigel, D., Lohmann, J.U., 2005. A gene expression map of Arabidopsis thaliana development. *Nat Genet* 37, 501-506.

Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M., Weigel, D., 2005. Specific effects of microRNAs on the plant transcriptome. *Dev Cell* 8, 517-527.

Schwarz, S., Grande, A.V., Bujdoso, N., Saedler, H., Huijser, P., 2008. The microRNA regulated SBP-box genes SPL9 and SPL15 control shoot maturation in Arabidopsis. *Plant Mol Biol* 67, 183-195.

Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa, H., Machida, C., Machida, Y., 2001. The ASYMMETRIC LEAVES2 gene of Arabidopsis thaliana regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. *Development* 128, 1771-1783.

Sessions, R.A., Zambryski, P.C., 1995. Arabidopsis gynoecium structure in the wild and in ettin mutants. *Development* 121, 1519-1532.

Siegfried, K.R., Eshed, Y., Baum, S.F., Otsuga, D., Drews, G.N., Bowman, J.L., 1999. Members of the YABBY gene family specify abaxial cell fate in Arabidopsis. *Development* 126, 4117-4128.

Smith, H.M., Hake, S., 2003. The interaction of two homeobox genes, BREVIPEDICELLUS and PENNYWISE, regulates internode patterning in the Arabidopsis inflorescence. *Plant Cell* 15, 1717-1727.

Spence, J., Vercher, Y., Gates, P., Harris, N., 1996. 'Pod shatter' in Arabidopsis thaliana, Brassica napus and B-juncea. *Journal of Microscopy* 181, 195-203.

Tiwari, S.B., Hagen, G., Guilfoyle, T., 2003. The roles of auxin response factor domains in auxin-responsive transcription. *Plant Cell* 15, 533-543.

Todesco, M., Rubio-Somoza, I., Paz-Ares, J., Weigel, D., 2010. A collection of target mimics for comprehensive analysis of microRNA function in Arabidopsis thaliana. *PLoS Genet* 6, e1001031.

Unte, U.S., Sorensen, A.M., Pesaresi, P., Gandikota, M., Leister, D., Saedler, H., Huijser, P., 2003. SPL8, an SBP-box gene that affects pollen sac development in Arabidopsis. *The Plant cell* 15, 1009-1019.

Venglat, S.P., Dumonceaux, T., Rozwadowski, K., Parnell, L., Babic, V., Keller, W., Martienssen, R., Selvaraj, G., Datla, R., 2002. The homeobox gene BREVIPEDICELLUS is a key regulator of inflorescence architecture in Arabidopsis. *Proc Natl Acad Sci U S A* 99, 4730-4735.

Wang, J.W., Czech, B., Weigel, D., 2009. miR156-regulated SPL transcription factors define an endogenous flowering pathway in Arabidopsis thaliana. *Cell* 138, 738-749.

Wang, J.W., Wang, L.J., Mao, Y.B., Cai, W.J., Xue, H.W., Chen, X.Y., 2005. Control of root cap formation by MicroRNA-targeted auxin response factors in Arabidopsis. *Plant Cell* 17, 2204-2216.

Warkocki, Z., Figlerowicz, M., 2006. Trans-acting short interfering RNAs. *Postepy biochemii* 52, 253-259.

Warthmann, N., Das, S., Lanz, C., Weigel, D., 2008. Comparative analysis of the MIR319a microRNA locus in Arabidopsis and related Brassicaceae. *Mol Biol Evol* 25, 892-902.

Williams, L., Carles, C.C., Osmont, K.S., Fletcher, J.C., 2005. A database analysis method identifies an endogenous trans-acting short-interfering RNA that targets the Arabidopsis ARF2, ARF3, and ARF4 genes. *Proc Natl Acad Sci U S A* 102, 9703-9708.

Woods, J.C., 2010. Analysis of FRUITFULL promoter motifs and their influence on valve expression during fruit development. Ms Thesis.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.W., Weigel, D., Poethig, R.S., 2009. The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. *Cell* 138, 750-759.

Wu, G., Poethig, R.S., 2006. Temporal regulation of shoot development in Arabidopsis thaliana by miR156 and its target SPL3. *Development* 133, 3539-3547.

Wu, S.L., 2012. Combining classical approaches and new technologies to identify and explore novel regulatory networks governing fruit development. Ms Thesis.

Xing, S., Salinas, M., Hohmann, S., Berndtgen, R., Huijser, P., 2010. miR156-targeted and nontargeted SBP-box transcription factors act in concert to secure male fertility in Arabidopsis. *The Plant cell* 22, 3935-3950.

Xu, L., Xu, Y., Dong, A., Sun, Y., Pi, L., Huang, H., 2003. Novel as1 and as2 defects in leaf adaxial-abaxial polarity reveal the requirement for ASYMMETRIC LEAVES1

and 2 and ERECTA functions in specifying leaf adaxial identity. *Development* 130, 4097-4107.

Yamaguchi, A., Wu, M.F., Yang, L., Wu, G., Poethig, R.S., Wagner, D., 2009. The microRNA-regulated SBP-Box transcription factor SPL3 is a direct upstream activator of LEAFY, FRUITFULL, and APETALA1. *Developmental Cell* 17, 268-278.

Yamasaki, K., Kigawa, T., Inoue, M., Tateno, M., Yamasaki, T., Yabuki, T., Aoki, M., Seki, E., Matsuda, T., Nunokawa, E., Ishizuka, Y., Terada, T., Shirouzu, M., Osanai, T., Tanaka, A., Seki, M., Shinozaki, K., Yokoyama, S., 2004. A novel zinc-binding motif revealed by solution structures of DNA-binding domains of Arabidopsis SBP-family transcription factors. *Journal of molecular biology* 337, 49-63.

Yant, L., 2012. Genome-wide mapping of transcription factor binding reveals developmental process integration and a fresh look at evolutionary dynamics. *American journal of botany* 99, 277-290.

Zhang, X., Zou, Z., Zhang, J., Zhang, Y., Han, Q., Hu, T., Xu, X., Liu, H., Li, H., Ye, Z., 2011. Over-expression of sly-miR156a in tomato results in multiple vegetative and reproductive trait alterations and partial phenocopy of the sft mutant. *FEBS Letters* 585, 435-439.

Zhu, Q.H., Helliwell, C.A., 2011. Regulation of flowering time and floral patterning by miR172. *Journal of experimental botany* 62, 487-495.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., Gruissem, W., 2004. GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant physiology* 136, 2621-2632.