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Uncovering new regulators of *Arabidopsis thaliana* fruit morphogenesis

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by

Quynh-Anh N. Mai

Committee in charge:

Professor Martin Yanofsky, Chair
Professor Nigel Crawford
Professor Robert Schmidt

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The thesis of Quynh-Anh N. Mai is approved and is acceptable in quality and form for publication on microfilm and electronically:

Chair

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DEDICATION

I dedicate this thesis to my little sister for putting up with me while I worked on this final chapter of my UCSD career. She also made me laugh a lot. Thanks, man!

TABLE OF CONTENTS

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ABSTRACT OF THE THESIS

Uncovering new regulators of *Arabidopsis thaliana* fruit morphogenesis

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Quynh-Anh N. Mai

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Professor Martin Yanofsky, Chair

The story of the network of genes that controls fruit patterning in *Arabidopsis* is still unfolding, and here we have seen familiar players take on different parts and we have identified new regulators that may play important roles. *Arabidopsis* fruit are patterned into three major regions: the valves, the replum, and the valve margin. Previous studies have shown that the *FRUITFULL* (*FUL*) and *REPLUMLESS* (*RPL*) genes are responsible for patterning the valve and replum, respectively. To identify new regulators, we screened for mutants that showed rescued replum development in the sensitized *rpl ful* double mutant background. We identified one suppressor (*m33*) that likely identifies a new fruit patterning gene as well as another suppressor (*m413*) that corresponds to the well-known floral homeotic gene *APETALA2* (*AP2*). Although previous studies have characterized the role of *AP2* during flower development and its regulation by *miR172*, little is known about its function during fruit morphogenesis. Here we used promoter-

GUS fusions to analyze the expression patterns of the five *miR172* genes in reproductive and vegetative tissues and found that *miR172* may be controlling AP2 activity in these tissues. In addition, we found that *FUL* may be an upstream regulator of *miR172* in the fruit. These studies have allowed us to incorporate the role for *miR172* into current models of fruit patterning. Although the final chapter to this story has yet to be written, our studies have added new insights that will undoubtedly contribute to the long-term goal of understanding the complex network of gene interactions that underlies fruit morphogenesis.

INTRODUCTION

Arabidopsis thaliana as a model organism for plant biology studies

Arabidopsis thaliana is a member of the Cruciferae (also called Brassicaceae) family of flowering plants (Redei, 1975) to which many dominant food crops such as broccoli, cauliflower, mustard, canola, etc. also belong. For the past 25 years, it has been widely preferred for use as a model organism in plant biology and development due to its many inherent characteristics that facilitate the study of several processes in plants. These characteristics include: life cycle and genomic organization that make it convenient for a number of the methods used in genetic studies; the small size of the mature plant and its seeds for ease of storage and growth of large populations, and large-scale mutagenesis (Meyerowitz, 1989); and the ease in which new genetic information can be introduced into it through transformation using *Agrobacterium tumefaciens*.

Studies using *Arabidopsis* have provided a plethora of available information that has been helpful in understanding and dissecting different processes in other plant species. For instance, the ABC model was revealed through the studies in *Arabidopsis*, and illuminated core biological rules for plant flowering in snapdragons (*Antirrhinum*) and other flowering species, as well as maize and many other crop species (Sommer et al., 1990; Whipple et al., 2004).

Wild-type fruit structure of *Arabidopsis*

The fruits are the harvested product of many crop species and have an important impact on diet and economy. Thus, understanding how the fruit is built would help us to

manipulate traits and quality of crop species for improving their agronomic importance. Furthermore, elucidation of the genetic and molecular interactions occurring in fruit development is critical for the construction of models that can be useful to improve fruit characteristics. Analysis using *Arabidopsis* has been providing a good platform to uncover important genes controlling the circuits that drive fruit development. To date, our lab has discovered a suite of regulatory genes that are required for fruit morphogenesis (Ferrándiz et al., 2000a; Ferrándiz et al., 2000b; Liljegren et al., 2000; Roeder et al., 2003; Dinneny et al., 2005; Liljegren et al., 2004; Østergaard and Yanofsky, 2004; Dinneny et al., 2005; Dinneny et al., 2006; Østergaard et al., 2006; Gremski et al., 2007; Crawford et al., 2007). It is likely that the functional understanding of fruit development genes in *Arabidopsis* will be directly transferable to crop plants, since most of the known fruit development genes from *Arabidopsis* have orthologs in a variety of eudicot and monocot crop plants (Dinneny and Yanofsky, 2004; Balanzá et al., 2006; Roeder and Yanofsky, 2006).

Many variations of fruit forms exist in the Brassicaceae family. The *Arabidopsis* fruit takes the more common form of the silique. It is a long cylindrical structure in which the gynoecium (the female part of the flower) is composed of two carpels (thought to be modified leaves; reviewed in Dinneny and Yanofsky, 2005) that are fused together (Ferrándiz et al., 1999; Dinneny and Yanofsky, 2005). At the top of the gynoecium lies the stigma, made up of a single layer of elongated papillar cells on which pollen attaches and germinates (Figure 1; Bowman et al., 1999; Ferrándiz et al., 1999; Balanzá et al., 2006; Crawford and Yanofsky, 2008). Below the stigma is the style, a solid cylindrical ring of vascular tissue that surrounds the transmitting tract through which pollen tubes

grow and move down the interior of the ovary (Sessions and Zambryski, 1995). Both the stigma and the style top the ovary, which consists of the valves, replum, and valve margins. Inside the ovary lie the ovules which develop from the placenta and are attached to the septum (the interior divisionary wall of the fruit) and the replum by funiculus (Bowman et al., 1999).

The two valves are the lateral walls of the ovary that surround and protect the developing seeds. Three identifiable regions of the valves are: the abaxial (outer) epidermal layer (or exocarp) of rectangular cells with interspersed stomata; the mesocarp consisting of three layers of chloroplast-containing cells; and the two layers of the endocarp, the lignified layer (en*b*) and the adaxial (inner) layer (en*a*) on the interior (Bowman et al., 1999; Ferrándiz et al., 1999). The valves are connected on both sides by the replum, which lies in the medial region of the fruit and divides the fruit in half. The replum on each side remains attached to the fruit after dehiscence (opening). Making up the boundaries between the replum and the valves are the valve margins (also known as the dehiscence zones. The valve margin can be divided into two territories, the lignified layer (on the valve side and connected to the en*b* layer of the valves) and the separation layer (on the replum side). When the fruit matures, enzymatic processes take place at the separation layer that degenerate the cell walls while, at the same time, the lignified layer help to create tension for the spring-like detachment of the valves from the replum and dehiscence occurs (Spence et al., 1996; Liljegren et al., 2000; Dinneny and Yanofsky, 2005).

Genes involved in fruit morphogenesis

Most of the studies carried out to uncover the regulatory genes controlling *Arabidopsis* fruit development have been performed in the laboratory of Prof. Martin F. Yanofsky. The resulting data of such studies have contributed to generate an initial genetic model to better understand fruit morphogenesis in *Arabidopsis.* Essentially, the model reveals that replum and valve gene activities have antagonistic functions and both negatively regulate valve margin genes from being expressed in their respective regions. In this way, the valve margin genes are allowed to be active in the narrow region of the valve-replum boundaries, forming the stripes that make up the valve margin (Dinneny et al., 2005; Alonso-Cantabrana et al., 2007). Next we present a brief review of the functions of some key genes controlling this process and how their genetic interactions contribute to define our current model.

A suite of genes collectively known as valve margin identity genes are all expressed in narrow stripes of cells at the valve-replum boundary where the valve margin later forms. These genes are *SHATTERPROOF1* and *SHATTERPROOF2* (*SHP1,2*), *ALCATRAZ* (*ALC*), and *INDEHISCENT* (*IND*) (Ferrándiz et al., 2000b; Liljegren et al., 2000; Rajani and Sundaresan, 2001; Liljegren et al., 2004; Dinneny and Yanofsky, 2005). Cell differentiation of both the lignified and the separation layers in the valve margin require the *SHATTERPROOF* genes, which are redundantly functioning MADS-box transcription factors (Ferrándiz et al., 2000b; Liljegren et al., 2000). Thus, *shp1,2* mutant fruits lack well-defined valve margins and do not dehisce. The *SHP* genes are also expressed in the ovules and style (Pinyopich et al., 2003; Favaro et al., 2003; Roeder and Yanofsky, 2006). *IND* encodes a basic Helix Loop Helix (bHLH) transcription factor

and is required for the development of both layers in the valve margin. Its loss-of-function (*ind* mutants) also causes indehiscence, but an even more severe loss of the valve margin phenotype than *shp* mutants (Liljegren et al., 2004). Like *IND*, *ALC* also encodes a bHLH transcription factor but its activity is only required for cell differentiation of the separation layer. So although they are indehiscent, *alc* mutants can shatter under pressure, and thus, observed to have a less severe phenotype than both *ind* and *shp* mutants (Rajani and Sundaresan, 2001). Both *IND* and *ALC* have been shown to be positively regulated by *SHP* in the valve margin (Ferrándiz et al., 2000a; Liljegren et al., 2000; Liljegren et al., 2004).

FRUITFULL (*FUL*) is a MADS-box domain transcription factor gene that is strongly expressed in the valves and also expressed in the style (Gu et al., 1998). In addition to its role in fruit morphogenesis *FUL* plays a role in floral meristem identity (Ferrándiz et al., 2000a). Before fertilization/anthesis (stage 11), the *ful* mutant gynoecium shows normal development (Ferrándiz et al., 2000a). After stage 11, *ful* mutant fruits show a severe reduction in the valve length while the replum continues to expand (Figure 2A). Due to the constraint of uneven growth between the replum and the valves, the replum adopts a zigzag arrangement (Ferrándiz et al., 2000a). Seed-crowding in the shortened siliques causes the valves to tear before seeds are mature (Gu et al., 1998). On the other hand, in *FUL* overexpressing plants, the exterior of the ovary is covered by valve cells. Based on these phenotypes, it would appear that *FUL* is required for the normal growth and differentiation of valve cells. However, the failure of valve cell development in *ful* mutants is due to the ectopic expression of valve margin genes in *ful* valves. This fact is most clearly illustrated by the observation that the tiny *ful* mutant

fruit is restored to nearly normal size and morphology in the *ful shp1 shp2 ind alc* quintuple mutant (Liljegren et al., 2004). So in fact, *FUL* acts to repress expression of valve margin genes in the valves to prevent these cells from adopting valve margin cell identity, and thereby allowing valve development (Roeder et al., 2003; Liljegren et al., 2004; Dinneny et al., 2005).

As *FUL* does in the valves, the BELL1 homeodomain transcription factor *REPLUMLESS* (*RPL*) negatively regulates valve margin genes by preventing their expression in the replum. Thus, in *rpl* mutants, replum development is impaired because valve margin genes become ectopically expressed in this tissue (Figure 2B). As expected, the removal of valve margin activities in *rpl* background restores replum development in a similar way to the restoration of valves in *ful* mutants (Roeder et al., 2003). In the context of the ovary *RPL* and *FUL* negatively regulate expression of valve margin identity genes in the replum and valve, respectively. Therefore, in the *rpl ful* double mutant, all cells of the external ovary acquire valve margin identity (Figure 2C; see below).

In recent years an additional layer of regulation controlling valve, replum and valve margin genes has been identified. 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) compose the *JAG/FIL* activity in the fruit (Dinneny et al., 2005). These genes are expressed in valves and valve margins and redundantly activate the expression of *FUL* and valve margin genes (Dinneny et al., 2005). *FUL* expression is more sensitive than that of the *SHP* genes to the loss of *JAG/FIL* activity, suggesting that *FUL* and *SHP* are activated by

different levels of this activity. More specifically, high levels of *JAG/FIL* activity in valves activate *FUL* expression, whereas this activity decreases towards the valve margin and is almost absent in the replum (Dinneny et al., 2005).

RPL interacts in the meristem with several class I KNOX genes, particularly *BREVIPEDICELLUS* (*BP*; Byrne et al., 2003; Smith and Hake, 2003; Bhatt et al., 2004). That interaction also seems to occur in the replum, since the *rpl bp* double mutants exhibit a very strong replumless phenotype, showing a synergistic interaction between both mutations. As expected, *BP* is expressed in medial tissues of the pistil that will give rise to the replum and positively control *RPL* expression in the replum (Alonso-Cantabrana et al., 2007). The expression of *BP* is, in turn, negatively regulated by *ASYMMETRIC LEAVES1* (*AS1*, MYB transcription factor) and *AS2* (LATERAL ORGAN BOUNDARY transcription factor; Byrne et al., 2000; Sun et al., 2002; Iwakawa et al., 2002). The direct interaction between *AS1* and *AS2* represses class I KNOX genes expression including *BP* (Guo et al., 2008). The absence of *AS1* (*as1* mutants) results in the misexpression of *BP* in the ovary and the fruits show an increase in replum size (Alonso-Cantabrana et al., 2007). In agreement with this, plants overexpressing *BP* show similar fruit defects. In addition to *BP*, *KNAT2* and *KNAT6* also belong to the class I KNOX homeodomain group. The expression of *KNAT2* and *KNAT6* has been found in the valve margin, and both *BP* and *RPL* seem to negatively regulate their activity (Ragni et al., 2008).

New strategies to find more genes involved in fruit development

In the current understanding of the fruit patterning network, *FUL* and *RPL* are key downstream regulators that negatively regulate valve margin identity to indirectly allow the development of valves and replum, respectively. Through genetic screens many other regulators of fruit patterning have been found (Eshed et al., 1999; Eshed et al., 2001; Roeder et al., 2003). However, some regulators may have been missed in screens because they may have more subtle phenotypes (Roeder and Yanofsky, 2006).

In the *rpl ful* double mutant fruit, the entire ovary surface is covered in valve margin cells, and there is no way to clearly distinguish the valves or the replum. With this phenotype, it is possible to identify more genes that affect valve margin development since the mutant would be able to suppress the phenotype and restore replum/valve development.

A former member of Prof. Martin Yanofsky's lab, Dr. Adrienne Roeder, performed a mutagenesis on *rpl-1 ful-1* seeds in the Landsberg *erecta* (Ler) background and *rpl-2 ful-2* seeds in the Columbia (Col) background using ethyl methanesulfonate (EMS) in order to identify new potential genes controlling fruit development (Roeder, 2005). Several suppressor plants were isolated by screening this population for restored replum development. Among them, the *rpl-1 ful-1 m33* mutant presented a clear replum growth restoration (Figure 2D). The initial mapping characterization of the *m33* mutation will be presented in the Results section.

Another mutant, *rpl-2 ful-2 m413*, was also found to have replum restoration (Figure 2E). Our unpublished data indicates that the *m413* mutation affects the microRNA (miR)-regulated floral homeotic gene *APETALA2* (*AP2*; Roeder, 2005; Ripoll

et al., unpublished). These results implicate *AP2* in the regulation of fruit development in *Arabidopsis* and also raise the matter of miR regulation in this process.

Previous studies on *APETALA2*

APETALA2 is the founder member of the AP2/EREBP (Ethylene Responsive Element Binding Protein) plant specific transcription factor family (Weigel, 1995; Riechmann and Meyerowitz, 1998; Shigyo et al., 2006). The AP2 protein contains two AP2 domains and together with five other members, *TARGET OF EAT1* (*TOE1*), *TOE2*, and *TOE3*, *SCHLAFMUTZE* (*SMZ*), and *SCHNARCHZAPFEN* (*SNZ*) form the AP2-like clade showing some functional redundancy as floral repressors (Schmid et al., 2003; Nöle-Wilson, 2005; Mathieu et al., 2009). Another important feature of *AP2* is that its activity is miR-regulated by *miR172* (Aukerman and Sakai, 2003; Chen, 2004).

The *AP2* gene is best known as a floral homeotic gene that specifies the A function of the widely recognized ABC model of floral organ identity (Coen and Meyerowitz, 1991). Although *AP2* is expressed in all four whorls, its function is proposed to work in the first and second whorls where it specifies sepal and petal identities and, at the same time, represses the C function gene *AGAMOUS* (*AG*; Jofuku et al., 1994; Weigel and Meyerowitz, 1994). This incongruity was resolved when Chen (2004) found that *miR172* activity was present in the third and fourth whorls blocking AP2 translation and preventing AP2 from repressing *AG* in these inner whorls (Chen, 2004). In concordance, *miR172* misexpression mimics flower defects found in *ap2* (Chen, 2004; Zhao et al., 2007).

In addition to the role that *AP2* has in flower development, recent works have identified that it controls the pool of stem cells in the shoot apical meristem through the *CLAVATA3* (*CLV3*)—*WUSCHEL* (*WUS*) pathway (Würschum et al., 2006) and also the floral meristem of the boundaries between the floral organs (Zhao et al., 2007).

Furthermore, it has been proven that *AP2* orthologs and *miR172* also play important roles in other species. For example, in maize, the *AP2*-like gene, *INDETERMINATE SPIKELET1* (*IDS1*) is required for the timely conversion of the spikelet meristem into the floral meristem (Chuck et al., 1998). In addition, *IDS1* and its related gene, *SISTER OF INDETERMINATE SPIKELET1* (*SID1*), repress the maize *AGAMOUS*-like orthologs within the lateral organs of the spikelet, which is similar to how *AP2* is required for floral organ fate in *Arabidopsis* (Chuck et al., 2007). Also similar to *AP2* in *Arabidopsis*, both *IDS1* and *SID1* are targets of *miR172* (Chuck et al., 2007; Chuck et al., 2008).

Through the *rpl ful* mutagenesis screening mentioned above, our lab discovered a new role for *AP2* in fruit development. *ap2* alleles have been shown to suppress the *rpl ful* mutation and restore replum development (our unpublished data), indicating that there are more avenues of *AP2* function in fruit development that need to be explored. One of them resides on the importance of the AP2 regulation by *miR172* during this process. In order to further explore this, several approaches were taken and the resulting data will be presented in the Results section.

MicroRNA biogenesis and their roles in plant development

The discovery of miRs as regulators involved in many important plant development processes is encouraging for the further exploration of how these processes have evolved and the mechanisms through which organ identity is conferred.

MicroRNAs are short (~21-24 nucleotides), non-coding RNA sequences that have been implicated in several biological processes through their action in modulating the activity/function of their target genes (Hake, 2003; Ke et al., 2003; Pasquinelli et al., 2005; Garcia, 2008; Voinnet, 2009).

In *Arabidopsis*, miRs are first transcribed by RNA polymerase II (Pol II) in the nucleus from intergenic regions (Figure 3A) as precursor-miR. These precursors adopt a secondary hairpin structure, which are then processed by proteins including Dicer-like1 (DCL1) to form mature dsRNA miR-segments containing a guide strand and a degraded strand (Park et al., 2002). After HEN1 methylates the duplex to protect them from degradation (Park et al., 2002; Yu et al., 2005), the transcripts are exported to the cytoplasm by the plant exportin 5 ortholog HASTY and other unknown factors (Park et al., 2005). The guide strand (after degradation of the other strand) is loaded onto Argonaute (AGO) proteins in the RNA-induced silencing complex (RISC) in order to carry out its function (Baumberger and Baulcombe, 2005; for review see Voinnet, 2009). The Argonaute proteins enable the activation of the miR they carry, and are believed to actually dictate the mode in which the miR regulates the target (Pasquinelli et al., 2005). It is believed that both animal and plant miRs repress target gene sequences one of two ways: transcript cleavage or translational repression (Figure 3B). Generally, if there is near-perfect base-pairing between the miR and its target sequence, the miR will repress

the target through the cleavage of the target mRNA; if there is imperfect pairing, translation of the target mRNA is inhibited (for review see Bartel, 2004; Dugas and Bartel, 2004). However, this paradigm already has an exception in *Arabidopsis*. *miR172* has nearly perfect base-pairing with its target sequence, *AP2*, and would be expected to regulate *AP2* expression through mRNA degradation. As mentioned earlier, it was found that *AP2* is actually inhibited at the translational level (Chen, 2004).

The studies performed in different model organisms, including plants, in recent years (Ochando et al., 2006; Kadener et al., 2009; Roush and Slack, 2009) have added substantial data to say that miRs are essential regulators of developmental processes. Although much effort has been focused on elucidating the mechanism of miR biogenesis and target gene regulation, relatively little is known and published about the upstream level of regulation of *miR* genes. In order to better understand the *miR172-AP2* interactions during fruit morphogenesis we used an approach based on the generation of promoter-driven *GUS* constructs for each *miR172* encoding gene. This approach might lead us to define the expression pattern and also to search for upstream regulatory elements of this *miR* family.

MATERIALS AND METHODS

Mapping strategy for *m33*

rpl-1 ful-1 m33 (Landsberg *erecta*, Ler, accession) seeds were crossed to *rpl-2 ful-2* in the Columbia (Col) ecotype (Roeder, 2005). The F1 progeny were allowed to self-fertilize in order to obtain the F2 mapping population. 90 F2 plants were selected based on rescued replum development. Simple Sequence Length Polymorphisms (SSLPs) were used as molecular markers to delimit the position of the *m33* mutation. See Table 1 for locations of markers and sequences of oligonucleotides used.

Cloning strategy and transgenic plants

To create transcriptional *β-glucuronidase* (*GUS*) *miR172* reporters, the promoter fragments of *miRNA172A* (At2g28056), *miRNA172B* (At5g04275), *miRNA172C* (At3g11435), *miRNA172D* (At3g55512), and *miRNA172E* (At5g59505) genes were each amplified and isolated using the proof-reading, high-fidelity Taq Polymerase (Phusion from New England Biolabs). The PCR products were subsequently cloned into the vector pGEM-T (Promega) after 3'A-tailing, and transformed into *Escherichia coli*. After sequencing, each promoter fragment was then excised by the enzymes listed in Table 2 and inserted into the T-DNA vector pJJGUS (Ripoll et al., 2006), which was also digested with the same enzymes. The integrity of the joints in pJJGUS was checked by sequencing. The resulting constructs, along with the pSOUP helper plasmid (Hellens et al., 2000), were transformed to *Agrobacterium tumefaciens* (AGL0 strain) by electroporation. For plant transformations we used the Col-0 accession and followed the

floral dip method (Clough et al., 1998). T1 transgenic plants harboring the corresponding *GUS* reporter were isolated by sowing seeds on MS plates containing 20 mg/ml Hygromicin.

GUS staining, histology, and microscopy

Inflorescence, seedlings, and fruit tissues were first treated with cold 90% acetone for 15 minutes, washed with DI water for 15 minutes at room temperature, infiltrated with *GUS* staining solution (25 mM sodium phosphate; 5 mM potassium ferrocyanide; 5 mM potassium ferricyanide; 1% Triton X-100, 2 mM X-Gluc) for 5 minutes, and incubated overnight at 37°C (Alonso-Cantabrana et al., 2007). Tissues were then fixed in FAA (50% ethanol : 3.7% formaldehyde : 5% acetic acid) for 2.5 hours, taken through an ethanol and Histoclear series, and embedded in Paraplast Plus. Tissue sections were generated at 8 μm thick using a Jung Biocut (Leyca) microtome. A standard dissecting scope with an adapted camera was used to take tissue whole-mount pictures. Slides were prepared and viewed as previously described (Roeder et al., 2003).

Total RNA extraction and quantitative RT-PCR

Total RNA was extracted from Col-0 wild-type and *ful-2* inflorescences (excluding opened flowers and fully-developed fruits) using the Qiagen Plant RNeasy Minikit according to provided instructions, and treated with DNaseI. 2.5μg of each sample of total RNA was used for single-stranded cDNA synthesis, which was done using oligo(DT) primer and SuperScript III Reverse Transcriptase from the SuperScript III First-Strand Synthesis System (Invitrogen). 2.5μl of single-stranded cDNA was used as a

template to perform quantitative RT-PCR (qRT-PCR) on the LightCycler 3.5 system (Roche) with the Quantifast SYBR Green PCR Kit (Qiagen). The relative changes in gene expression were calculated relative to *ACTIN2* using the $2^{-\Delta\Delta CT}$ method (Ripoll et al., 2009). Each experiment was done three times using different biological replicates. The averages and standard deviation were calculated in Microsoft Excel. See Table 3 for sequences of oligonucleotides used for qRT-PCR.

Site-directed mutagenesis

Scanning of the *miRNA172* genes promoter sequences using PLACE program (http://www.dna.affrc.go.jp/PLACE/) revealed that each promoter contained at least one putative CArG box for the binding of MADS domain transcription factors (Figure 5). Promoter fragments cloned into pGEM-T, obtained through the strategy outlined above, were used as the precursors for the specific mutagenesis of these CArG box motifs with the primers listed in Table 4. Also indicated in Table 4 are the mutations that were made to each CArG box sequence. The presence of the mutations was corroborated by sequencing and the promoters were then excised and cloned into pJJGUS as described earlier.

RESULTS

I. *m33* suppresses the *rpl ful* fruit phenotype and maps to Chromosome II

As mentioned in the Introduction, in *rpl ful* mutants, the entire ovary surface is covered in cells with valve margin identity, and the replum as well as the valves are absent. This sensitized background provides a good canvas on which to screen for suppressors that can rescue replum development and seek for genes that interact to control the formation of this territory. One of the suppressors isolated was the *m33* mutant. The *m33* mutation restores replum development in *rpl-1 ful-1* background, in which the replum adopts a protruded and zigzag shape due to the reduced fruit size (Figure 2; Roeder, 2005).

We used a map-based cloning strategy to identify and characterize the *m33* mutation and, subsequently, the affected gene. We generated an F2 mapping population by crossing *rpl-1 ful-1 m33* (Ler) to *rpl-2 ful-2* (Col) and isolated the plants showing the suppressed phenotype of rescued replum development. We used SSLP molecular markers to develop our cloning approach. Strong linkage was found to the marker CIW2 which is located at 9.95 cM on Chromosome II. The recombination frequency between the *m33* mutation and CIW2 was 16.4%. Therefore, the location of *m33* is most likely within the region around CIW2, indicated by the red box in Figure 4. This region of Chromosome II contains several interesting candidate genes including *CLE16* (*CLAVATA3*-like gene 16). Through the CLE-related work our lab is doing, we know that *CLE16* shows valve margin expression (our unpublished data). However, there has not been a mutant phenotype detected for *cle16* mutants, which may be due to redundancy with other *CLE*

genes that are also expressed at the valve margin (our unpublished results). Nevertheless, mutation of the *CLE16* gene would resonate well with the fact that removal of valve margin genes rescues replum development in *rpl ful* background. Currently, we are sequencing *CLE16* in the *rpl-1 ful-1 m33* background. See Table 1 for recombination frequencies found between *m33* and other markers on Chromosome II.

II. Analysis of the transcription of *miR172* family members in *Arabidopsis*

As a result of our screening for suppressors of *rpl ful* "replumless" phenotype, we identified that one of such suppressors was allelic to *ap2*. As mentioned in the Introduction, the *AP2* gene is best known for its role in floral organ identity and that its activity is postranscriptionally miR-regulated by *miR172* (refer to the Introduction). However, neither *AP2* nor *miR172* involvement in fruit morphogenesis and patterning have been carefully examined to this date.

A member of Prof. Yanofsky's Lab is dissecting the role of *AP2* during fruit morphogenesis, and has demonstrated a functional requirement for *miR172* in valve development (Figures 5D and 5H). A *miR172*-resistant version of *AP2* (*AP2m*) was specifically expressed in valve tissue using the *FUL* promoter (*FUL>>AP2m*). The resulting fruit had impaired valve development and resembled those of *ful* mutants (Figure 5 and our unpublished data).

Previous work reported that *miR172* is expressed in stamen and carpel primordia, but did not provide details of either expression or function during fruit development (Chen, 2004). Thus, to evaluate *miR172* expression in the fruit, we developed *GUS*-based reporter lines for all five *miR172* genes (*miR172A::GUS, miR172B::GUS,*

miR172C::GUS, miR172D::GUS, miR172E::GUS) and we examined their patterns. The use of *GUS* constructs to report *miR* expression patterns has been successfully proven and demonstrated by others. Raman et al. (2008) developed this strategy to analyze the expression pattern for the *miR164* gene family (*miR164A*, *miR164B* and *miR164C*) in *Arabidopsis*. The putative 5' regulatory sequence of each *miR172* gene was fused to *GUS* gene reporter of the pJJGUS T-DNA vector (Ripoll et al., 2006) as described in the Materials and Methods section.

In the following sections we will describe the expression patterns obtained for each *miR172* reporter line in reproductive (pistils and fruits) and vegetative tissues. Several transgenic lines were obtained for each *miR172* reporter constructs. *GUS* activity was checked in pistil tissues in both pre- and post-anthesis stages as well as 3- and 7-days old seedlings. For each line, the expression patterns obtained in T1 generation were corroborated in T2 and T3 generations. For each *miR172* reporter, we identified strong, moderate, and weak expressing lines among the ones isolated.

II.1. *miRNA172* expression patterns in *Arabidopsis* reproductive tissues

For *miR172A::GUS*, twenty T1 lines were obtained. Pistils in both pre- and post-fertilization stages were stained for *GUS* activity. The *GUS* expression patterns were consistent among most of the lines and corroborated in the next generations (T2 and T3 lines). In all the lines the signal was found in the adaxial region of valves, mostly likely corresponding to the inner layers of the mesocarp and the endocarp (en*a* and en*b*, Figure 1). The expression in these regions persisted through all the stages during fruit maturation (Figures 7A-C). Upon close observation, no signal was found in the replum

of young pistils. However, weak *GUS* expression was present in the inner (adaxial) part of the replum where the vasculature is located. Nevertheless, it is possible that this signal is background because of its weakness. The outer-most cell layers of the replum at that stage show no signal (Figure 7A-C). *miR172A*::*GUS* expression was also found in ovules after fertilization and developing seeds (Figures 6F and 7A-C). Although the funiculus is a closely related structure to the ovule (Crawford and Yanofsky, 2008), no expression was found there (white arrowheads in Figures 7A and 7B). Whole mounts and longitudinal sections showed low *GUS* activity in the style (Figure 6A and 6F). The locations of *GUS* expression were consistent in all lines examined.

Transgenic lines for *miR172B::GUS* were more difficult to generate and we only isolated ten T1 lines. Most of these lines showed very strong *GUS* activity. The expression pattern of *miR172B::GUS* in fruits was very similar to that of the *miR172A::GUS* in that it was seen in the same fruit territories (Figures 6B and 6G). However, there was comparatively stronger signal expression of *miR172B::GUS* than *miR172A::GUS*. Expression levels in the valves were consistent throughout all the valve layers and remained so for both pre- and post-fertilization stages. *miR172B::GUS* signal was constricted to the adaxial replum before and after fertilization and no signal was detected in the outer-most layers of the replum. As described for *miR172A*, *miR172B::GUS* signal was also detected in ovules during development. However, after fertilization, ovule expression decreased and gradually ceased in the later stages when the ovules become seeds (Figures 7D-F). No funiculus signal was detected for *miR172B::GUS*. Later stages also provided an opportunity for better clarification of specific *miR172B::GUS* expression with medial-lateral cross-sections clearly showing no

activity in the valve margins (white arrowheads in Figure 7F). Furthermore, when taking into account the high levels of expression observed for these reporters, we cannot rule out that signal in the valve margins at earlier stages was simply background.

Sixteen transgenic T1 lines were isolated for the *miR172C::GUS* reporter. *GUS* activity was checked in T1 lines and corroborated in the next T2 and T3 generations. For this reporter, all lines showed specific and strong *GUS* activity in all the layers of the valves (Figures 7G-I). However, after fertilization, the valve signal gradually decreased and became restricted to the inner layers of the valves (en*a* and en*b*). This was also observed for the *miR172A* and *miR172B* reporters. Before fertilization, weak *miR172C::GUS* signal was found in the inner (adaxial) replum that was significantly diminished by stage 16-17 (Figures 7G-I). Weak expression in the in the style (most likely the vascular tissue) was detected (Figures 6C and 6H). No ovule or funiculus expression was detected for this reporter line. Keeping the general *miR172C::GUS* expression pattern in mind, we might consider *miR172C* as the valve-specific *miR172*.

Similar to what occurred for the *miR172B::GUS* lines, T1 lines for the *miRNA172D* reporters were difficult to generate and only nine were isolated. Although there were some differences in the levels of expression, all the *GUS* lines examined for *miR172D* showed very consistent *GUS* patterns that were corroborated in T2 and T3 generations. In transformants showing high *GUS* activity, strong expression was very specifically localized to the ovules and the tissue surrounding the vascular bundle in the inner replum (Figures 7J-L). Relatively low activity was observed in the style in longitudinal sections and whole mounts pictures for *miR172D::GUS* fruits (Figures 6D, 6I, and 7J-L).

For the *miR172E::GUS* transgenic reporter, twenty-three T1 transformants were obtained and checked for *GUS* signal. T2 and T3 lines were also stained to confirm the patterns observed. In whole-mount pictures and longitudinal sections we consistently found style expression (Figures 6E and 6J). Although not observed in whole mount or longitudinal sections, pistil cross-sections revealed that *miR172E*::*GUS* was expressed in the inner layers of the valves (Figures 7M-O). Once fertilization took place *miR172E* valve expression decreased and became restricted to the vascular tissue (white arrowheads in Figures 7M-O). In addition, signal was present in the adaxial replum (where the vascular tissue is present) throughout all fruit stages.

In summary, all five *miR172* reporters shared expression domains in some ways. With the exception of *miR172D* most of the *miR172* reporters seemed to be present in the inner layers of the valves (*miR172C* reporter was expressed in all valve cell layers). Furthermore, all of the *miR172* reporters were found to be expressed in the adaxial region of the replum where the vasculature is located. Ovule expression appeared to be specific for *miR172A, miR172B* and *miR172D* (see Table 5 for a summary of *GUS* activity for all transgenic lines). The overlapping expression for all five reporters in the ovary indicates that the *miR172* target *AP2* would be active in the abaxial cell layers of the replum and valve margin. This result is not surprising since *ap2* fruits show mutant phenotypes in those fruit territories (our unpublished data). On the other hand, we know that several transcription factors involved in fruit patterning are present in the regions where the *miR172* transcription was detected (see Introduction). So, *miR172* might putatively be under the control of such transcription factors during fruit morphogenesis. Further experiments to get more insights on this regulation will be presented in later sections.

II.2. *miRNA172* expression patterns in *Arabidopsis* vegetative tissues

Recent studies have found that in addition to specific roles in flower and now fruit development, *AP2* is also involved in regulating the stem cell niche in the shoot apical meristem (SAM) of *Arabidopsis* (Würschum et al., 2006). Furthermore, several genes controlling leaf development tightly control fruit development as well (Roeder et al., 2003; Dinneny et al., 2004; Dinneny et al., 2005; Alonso-Cantabrana et al., 2007). Therefore, in addition to analyzing *miR172* promoter-driven *GUS* expression in the reproductive tissues, we also checked the expression pattern of each *miR172* reporter in seedlings (during the vegetative phase) to further understand *miR172*'s role in the regulation of *AP2* during plant development.

T2 and T3 transformants were selected on hygromicin, and *GUS* expression was examined in seedlings 3 and 7 days after germination (dag). In 3 dag seedlings no expression was found at the SAM for any of the reporters. With the exception of *miR172D*, *miR172* was present in the region located below the SAM and also and near the flanks of the SAM (Figure 8). The same patterns were observed in 7 dag seedlings. However, at that stage, *miR172D::GUS* was barely detected below the SAM. Surprisingly, we found *GUS* signal for *miR172C* at low levels at the tip and high at the center of the SAM (Figure 8L). More interestingly, *GUS* signal was found for all *miR172*'s in developing leaf primordia, with the exception of *miR172D* (Figure 8N and 8P).

These overall results, previous data from Würschum et al., 2006, and our unpublished data indicate that *miR172* and *AP2* most likely play a role during vegetative development. Our lab and others (Würschum et al., 2006) have found that *AP2* is able to

control the expression of some meristematic genes. The absence of *miR172* at the SAM in early stages (3 dag) would indicate the presence of *AP2* activity for controlling early events in meristem development and, later, organ formation.

II.3. Upstream regulators of *miR172* expression during fruit morphogenesis

As presented above, the *miR172* genes shared fruit expression domains with some of the most important regulators in fruit morphogenesis. The MADS-box transcription factor *FUL* is specifically expressed in carpels and valves (Gu et al., 1998; Ferrándiz et al., 2000b). Strikingly we have also found valve expression for *miR172A::GUS*, *miR172B::GUS* and *miR172C::GUS*. This raises the possibility that the transcription for miR172A, B and C could be under FUL control. Thus, we started several approaches to rule it out. First, we crossed each reporter to loss- (*ful* mutants) and gain-of-function (*35S::FUL*) *FUL* backgrounds. On the other hand, we also studied the expression levels for such *miR172*'s in the same mutant backgrounds by qRT-PCR. This technique has been successfully used in previous works to report changes in *miR* expression levels in several organisms including *Arabidopsis* (Schmittgen et al., 2004; Chambers and Shuai, 2009; Pant et al., 2009; Yang et al., 2009, among others).

Taking advantage of both the *FUL* overexpression line and the fact that *miR172::GUS* reporters are dominant markers, we crossed our reporter lines to plants in the *35S::FUL* background to test whether *FUL* was able to activate the expression of these *miR172* reporters. As depicted in Figure 9B and 9C, *miR172C::GUS* expression is induced in *35S::FUL* background. The results for *miR172A* and *B* reporters will soon be obtained and we expect to see similar behavior for each one.

The reduction in the expression for *miR172A, B* and *C* in *ful* loss-of-function mutants would be in consonance with the result above mentioned. To pursue this aspect, we crossed the corresponding reporter lines to plants in the *ful-2* mutant background. After verifying the presence of the transgene by *GUS* staining, the F1 plants were self-fertilized to obtain the F2 progeny. We will soon start to identify *ful* plants carrying each corresponding transgene and study their expression patterns.

In the meantime we decided to study the relative *miR* precursor levels for miR172A, B and C in *ful* mutants to compare them to the wild-type reference Col. *ful* fruits display a dramatic phenotype (Gu et al., 1999; Ferrándiz et al., 2000a). However, before anthesis *ful* and wild-type pistils look very similar (Ferrándiz et al., 2000a; our unpublished data). Therefore, we extracted total RNA from inflorescences (and removed opened flowers) from both *ful* and Col plants. As shown in Figure 9A there was a five-fold reduction of miR172B precursor in *ful*, compared to wild-type. In the case of *miR172C* the difference was even more dramatic, showing a fourteen-fold down-regulation of the precursor. The qRT-PCR results for *miR172A* were inconclusive because we had difficulty amplifying its precursor in both *ful* and wild-type tissue. Although *miR172A::GUS* is clearly visible from our *GUS* assays, perhaps the levels of its precursor are not sufficient to be detected by this technique. However, we are also using several alternative pairs of primers to test for the amplification of this miR172A precursor.

As presented above, some of the *miR172* reporters showed clear and consistent ovule and seed expression (*miR172A, B* and *D*). The AG-clade MADS-box transcription factors *SEEDTICK* (*STK*), *SHP1* and *SHP2* collaborate during fruit development for

proper ovule/seed development (Pinyopich et al., 2003; Favaro et al., 2003). Following this thread, we wondered whether such genes were, in some fashion, regulating the expression of this set of *miR172*'s in ovules/seeds as *FUL* most likely does in the valves. Thus, we initiated several experiments to rule out this possibility. We have crossed the *GUS* reporters to *stk* and *shp1,2* mutant backgrounds and got some of the F2 populations. At this moment we are trying to identify the mutant plants harboring the *GUS* reporters among the F2 individuals. In addition, qRT-PCR results will soon be obtained to test whether there is down-regulation of the *miR172*'s in *stk* and/or *shp1,2* backgrounds.

II.4. Analysis of the *miR172* promoter sequences

In *Arabidopsis* as well as in other organisms, the vast majority of the regulatory elements controlling morphogenesis encode for transcription factors that fine-tune gene expression through the regulatory sequences (binding motifs) present in the promoter of their targets. Be that as it may, little is known and published about the regulation of the transcription of the regulatory *miR* genes in *Arabidopsis* (Megraw et al., 2007). Thus, the identification and analysis of transcription factor binding motifs on the putative *miR* promoters could provide new clues to identify and study possible transcription factors controlling *miR* transcription.

One family of transcription factors that plays crucial roles during *Arabidopsis* development is the MADS-box gene family (Theissen et al., 2000). MADS domain transcription factors bind to a family of closely related DNA motifs called CArG box (Dolan and Fields, 1991; Treisman, 1992; Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Shiraishi et al., 1993; Savidge et al., 1995; Zachgo et al., 1995; Davies et al., 1996;

Huang et al., 1996; Mizukami et al., 1996; Riechmann et al., 1996; Tilly et al., 1998; Lauri et al., 2006; Kaufmann et al., 2009). As mentioned in the Introduction, members of this family of MADS-box genes control different aspects of fruit development. For instance, this is the case for the MADS-box gene *FUL* controlling valve development, and *SEEDSTIK* (*STK*), which controls ovule development. *SHP* genes are also MADS-box genes that control valve margin formation and ovule growth (Ferrándiz et al., 2000a; Liljegren et al., 2000; Pinyopich et al., 2003).

In this context, we followed an active approach to identify CArG motifs on the promoter sequences of each *miR172*. We used PLACE (Higo et al., 1999) and found the presence of at least one CArG box on each promoter (Figure 10). In order to test how important they are, we are following an approach based on the mutagenesis of those motifs, and we will then check the resulting expression patterns for the corresponding reporter. Currently, we are mutagenizing such elements for each promoter and generating the reporter *GUS* lines. By checking the expression pattern of the reporter of each *miR172*, we will be able to see if there is an effect on the expression of the regulatory genes. If an effect does occur on expression, we can combine this and previous data to suggest that MADS-box genes control *miR172* in the fruit. Further experiments can be also done in the future to test whether there is, in fact, binding to such CArG boxes.

DISCUSSION

m33 affects gene on Chromosome II to suppress *rpl ful* phenotype

The *m33* mutation was isolated in a mutant screen generated with the *rpl ful* sensitized background, and showed a rescued replum development phenotype. Mapping was done to determine that this mutation is located in the region above the centromere of Chromosome II. In this region also lies an interesting gene, *CLE16* (*CLAVATA3*-like gene 16).

CLE gene family members are structurally related to *CLV3* (the founder member) and encode plant signaling peptides (Cock and McCormick, 2001; Sawa et al., 2006; Mitchum et al., 2008). Although not much more is currently known about the role(s) of *CLE* genes in *Arabidopsis*, numerous studies have been done on *CLV3*. *CLV3* functions in a feedback loop regulating *WUSCHEL* (*WUS*) to maintain proper levels of stem cells in the shoot apical meristem (Clark et al., 1995; Schoof et al., 2000; Baurle and Laux, 2005). Our unpublished data shows that *CLE16* is expressed in the valve margin. However, its specific function(s) has been difficult to identify since *cle16* mutant fruits do not show any phenotype. One possible explanation for this is that it may act redundantly with other *CLE* genes also expressed in the valve margin (our unpublished data). According to the current model, proper development of the replum is indirectly controlled through the inhibition of ectopic expression of the valve margin genes by regulators in that territory. If *CLE16* is in fact a valve margin gene, it would be possible for the *m33* mutation to rescue replum development in *rpl ful* by affecting the expression of *CLE16* in that territory. Accurate sequencing to identify if there is a mutation in

CLE16 in the *rpl ful m33* mutant would reveal that *CLE16* might be a new regulator involved in regulating the patterning along the medial-lateral axis (valve—valve margin—replum).

Fruit development is also impacted by the microRNA-regulated gene *AP2*

From the same screen, it was found that another suppressor, *m413*, also showed rescued replum development in the *rpl ful* background. Single mutant (*m413*) flowers showed same phenotype as *ap2* flower with carpelloid identity in the first whorl (Drews et al., 1991; Jofuku et al., 1994). Sequencing of the *m413* mutation and complementation test revealed that it is allelic to *ap2*. Although it has been suspected that *ap2* mutants would have fruit defects (Bowman et al., 1991b), these defects have not been analyzed until recently. We found that indeed *ap2* fruit mutants show abnormal phenotype in the replum and valve margin (our unpublished data). Thus, we are beginning to understand that *AP2* has further roles that include the development of fruit tissues. Following the thread of previous work that our lab has done to elucidate much of the genetic network controlling fruit patterning, we are developing new strategies to dissect *AP2* function(s) in the fruit. At the same time, we have been able to complement these studies with our work on miR regulators in *Arabidopsis*.

As we have seen before, *miR* regulation of *AP2* has been found to play a key role in flower development. The misexpression of a *miR172*-resistant version of *AP2* resulted in dramatic floral phenotypes. However, no alterations were identified in the miR-regulated version (Chen, 2004; Zhao et al., 2007). Thus, *miR172* functions to tightly control *AP2* activity in flowers (Chen, 2004). This regulation, however, had not yet been

studied in fruit development. Following this method, our lab created a miR-resistant *AP2* (*AP2m*) and expressed this in valve domains by using *FUL* promoter (transactivation system, Moore et al., 2006; *FUL>>AP2m*). Misexpression of this resulted in impaired fruit development, with siliques showing dramatic reduction of the cell size in the valves as was previously described for the *ful* mutant (Figure 5). This indicates the need of miR regulation of *AP2* for proper fruit development. In addition, it also indicates that indeed, *AP2* is somehow able to impair *FUL* activity altering the normal valve growth. In light of this finding, we wondered whether any of the *miR172* species was present in valves.

We used an approached based on promoter-driven *GUS* constructs to identify the presence of the expression of *miR172* genes in fruits. All five *miR172* reporters were active in fruits. However, none of the *GUS* reporters was present in neither the epidermal replum cells or in the valve margin, indicating that the *miR172* target *AP2* would be active in these regions. In actuality, *ap2* fruits are affected in both replum and valve margin formation (our unpublished data). To further see if the *AP2* protein is in fact expressed in the regions where *miR172* is absent (*in vivo*), translationally-fused *GUS* or GFP reporter constructs could be made with *AP2*. Overall, it seems that *miR172* controlling *AP2* activity in these domains is just as important for normal development of the fruit as *miR172* regulation of *AP2* is for flower development.

Upstream regulators of *miR172*

Although the *miR172* reporters shared expression domains, some were preferentially expressed in specific tissues. Only the *miR172C* reporter was present in the outer layer of the valves and its activity was found strongly and primarily in the valves.

Although expressed in low levels and restricted to the inner layers, *miR172E* also seems to be specifically present in valve tissue. *miR172A* and *miR172B* both showed high reporter activity in the valves as well, but they were also strongly expressed in the ovules, where the *miR172D* reporter was also predominantly expressed. The strength and specificity of expression of each *miR172* in certain regions of the siliques suggests that each *miR172* might be associated with the function of particular set of genes involved in the correct formation of such regions. The analysis of the expression patterns of the different *miR172*'s by using *GUS* reporters in different fruit domains not only provides a gain of knowledge about *miR172-AP2* interplay in fruit development. They also might help to unveil the upstream elements that would be regulating the expression of the *miR172*'s in fruits.

In the current model, several transcription factors involved in fruit patterning are present in the regions where the *miR172* transcription was detected (see Introduction and Results; Gu et al., 1998; Ferrándiz et al., 2000a; Pinyopich et al., 2003; Favaro et al., 2003). Several of these elements are MADS-box transcription factors that bind to CArG boxes motifs in target promoters to regulate expression of those genes (Ferrándiz et al., 2000b; Liljegren et al., 2000). Using PLACE (Higo et al., 1999) to look for promoter elements in the *miR172* promoters, we found that each *miR172* promoter contained at least one CArG box domain (Figure 10). This presented the possibility that some of these transcription factors may be regulating the expression of the *miR172* genes in certain regions during fruit morphogenesis.

Because *miR172B* and *miR172C* reporters showed high and clear expression in the valves, where the MADS-box gene *FUL* plays an important role, we examined the

levels of *miR172B* and *miR172C* precursors in *ful* mutants (through sq and qRT-PCR) to determine if *FUL* controls their expression. In the absence of *FUL*, the expression of both *miR172B* and *miR172C* was dramatically down-regulated compared to levels in wild-type fruits (Figure 9A). However, the levels were not completely abolished, indicating that there may be other factors involved besides *FUL*. This is reasonable, considering that—first, in addition to *FUL*, other genes are also controlling valve formation (Dinneny et al., 2004 ; Alonso-Cantabrana et al., 2007)—and secondly, we saw expression in the ovules and the style for *miR172B::GUS* and in the style for *miR172C::GUS* where other transcription factor could be regulating their expression. According to our *GUS* assays, *miR172A::GUS* was also seen in the valves, but we were not able to reliably amplify this precursor in both *ful* and wild-type tissues perhaps because the endogenous levels of miR172A precursor are not high enough for detection by qRT-PCR. To support these findings, we are crossing (and will soon analyze) these reporter lines (*miR172A*::*GUS*, *B* and *C*) to *ful*.

We also took another approach wherein we crossed the *miR172::GUS* reporter lines to *35S::FUL* to see whether overexpression of *FUL* was able to activate the transcription of these *miR172*'s. So far in F1 progeny of the *35S::FUL* cross with *miR172C::GUS*, expression of *miR172C* reporter activity was found in nearly all tissues studied, and at high levels, showing that higher levels of *FUL* led to the up-regulation of *miR172C*. More accurate confirmation of the higher levels in of this could be done through measuring the miR precursor levels in the *35S::FUL* plants by qRT-PCR, which we are currently working on. We have also crossed the other two relevant valve *miR172* reporter lines to *35S::FUL* lines and expect similar results. Overall, it appears that *FUL*

positively regulates *miR172* genes since the levels of *miR172* expression clearly correspond to levels of *FUL*.

In light of this, it is possible to postulate that perhaps other MADS-box transcription factors may also regulate *miR172* expression since some of the *miR172* genes have expression patterns in domains that overlap the regions where these known regulators act. *SHP1,2* and *STK* are MADS-box genes that are highly expressed in the ovules and have been found to act redundantly for the development of this tissue (Pinyopich et al., 2003; Favaro et al., 2004). *miR172A*, *B*, and *D* could be regulated by these genes because they show prominent expression specifically in the ovules. Another key regulator and MADS-box gene, *AG*, is known for conferring the carpel identity during floral development (Yanofsky et al., 1990; Bowman et al., 1991a; Drews et al., 1991). *AG* has also been shown to regulate the expression of both *SHPs* and *STK* for ovule development (Pinyopich et al., 2003; Favaro et al., 2004).

Further studies are currently being carried out to determine if perhaps *SHPs*, *STK*, and somehow *AG* are also involved in regulating *miR172* transcription. Moreover, the *miR172* reporter lines have been crossed to *shp1,2* and *stk* loss-of-function, as well as *ag*/+ (since *ag* mutants lack carpels). We will also check these levels in the mutant backgrounds using qRT-PCR. Since these MADS-box transcription factors bind to CArG box domains, we are also working on seeing if there is an effect when CArG box motifs are mutated in the *miR172* promoters of our *GUS* reporters. If, in fact, these factors regulate *miR172* expression, the removal (or alteration) of such motifs would be in line with the *GUS* expression patterns obtained in the *shps*, *stk* or *ag* loss-of-function.

It cannot be ruled out that *miR172* may be also be subjected to negative regulation as well. Seeing that *miR172* reporter activity was not present in the replum and the valve margin helped to understand the role of *AP2* in fruit development. Thus, it might be possible that factors present in these domains would function to inhibit *miR172* expression for proper patterning. Since *RPL* is expressed in the replum (Roeder et al., 2003), it is possible that *RPL* may play this role to control *miR172* expression. As presented earlier, more regulators are currently being discovered that may also function in this territory for proper replum development. Perhaps once they are identified, further postulations may be made in order to more precisely understand how *miR172* fits into the current genetic model.

Genetic regulation between *FUL*, *miR172*, and *AP2* during fruit development

So far, this study has elucidated that, in addition to flower development (Chen, 2004; Zhao et al., 2007), *AP2* regulation by *miR172* also plays an important role in fruit patterning. Our lab's efforts have also revealed that *AP2* is able to somehow regulate *FUL* activity, with important consequences to the identity of cells in valve tissues. And finally, results have also shown *FUL* is most likely a positive regulator of *miR172* expression in the valves. It is clear that these three factors—*miR172*, *AP2*, and *FUL*—interact with each other in a feedback loop fashion. This type of genetic regulation has been seen before between miRs and other transcription factors (Johnston et al., 2005; Odom et al., 2006; Crews and Pearson, 2009). However, the analysis of *miR* regulation with further studies, such as what we began here, might also uncover new connections among the genes participating in the network controlling fruit development.

miR172 involvement during the vegetative phase

Several of the key regulators that have been identified in the fruit development network also have roles controlling many aspects of the plant during the vegetative phase (Ferrándiz et al., 2000b; Alonso-Cantabrana et al., 2007; Melzer et al., 2008; Wang et al., 2009). Since it is widely believed that carpels are modified leaves (Goethe, 1870; reviewed in Dinneny and Yanofsky, 2005), understanding how these genes function in the SAM and leaves has provided insight into their function in fruit development (Girin et al., 2009). It was recently found by Würschum that through the *CLV3-WUS* pathway, *AP2* also has a role in regulating the stem cell niche in the SAM. Seeing that the function of *AP2* extends to this vegetative phase in plant development, it might be possible that *miR172* would also be regulating *AP2* activity in vegetative tissues.

Our data showed that *miR172* is not expressed early on in the SAM as *AP2* is, but was seen primarily in the regions below the SAM and the leaf primordia. It is possible that the presence of *miR172* in these regions is somehow important in controlling the activity of *AP2* in order to maintain proper activity of the meristem. We may be able to show this with further studies, such as expressing a microRNA-resistant version of *AP2* in these regions.

Overall, the results we obtained showed that investigation using these approaches was an ideal way to elucidate the expression pattern of *miR172* in fruit tissue to ultimately better grasp how *miR172* and other *miR*'s may be involved in the genetic framework for fruit morphogenesis in *Arabidopsis*. Many *Arabidopsis miR*'s are conserved among flowering plants (Axtell and Bartel, 2005), and they have been found to function in a myriad of developmental processes such as flowering time, floral

development, organ polarity and vasculature development, among others (McConnell et al., 2001; Aukerman and Sakai, 2003; Emery et al., 2003; Palatnik et al., 2003; Achard et al., 2004; Chen, 2004; Juárez et al., 2004; Mallory et al., 2004; McHale and Koning, 2004; Zhong and Ye, 2004; Kim et al., 2005; Millar and Gubler, 2005), and recently, fruit fertilization (Wu et al., 2006). Now our lab has discovered that *miR*'s are also involved in the regulation of fruit patterning along the medial-lateral axis. Additionally, using our *GUS* reporters, we have also found that *miR172* is also present in vegetative tissues, which opens up new areas to explore.

APPENDIX A: FIGURES AND FIGURE LEGENDS

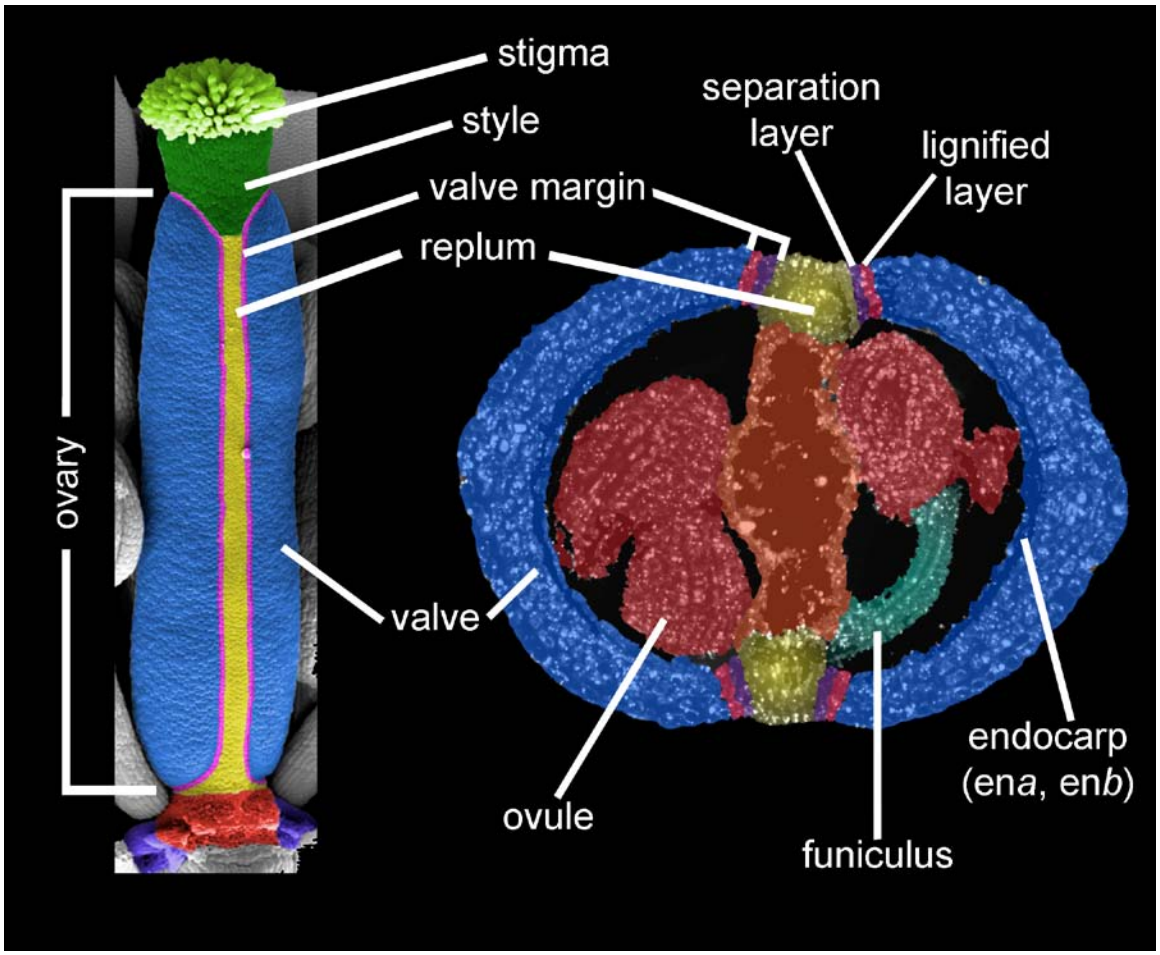


Figure 1. Organization of *Arabidopsis thaliana* fruit tissues.
On the left is a scanning electron micrograph (SEM) of a wild-type fruit gynoecium with different parts labeled. On the right is a medial-lateral cross section of a mature wild-type fruit with different parts of the gynoecium labeled. The valve margin is composed of both the lignified layer on the valve side and separation layer on the replum side.

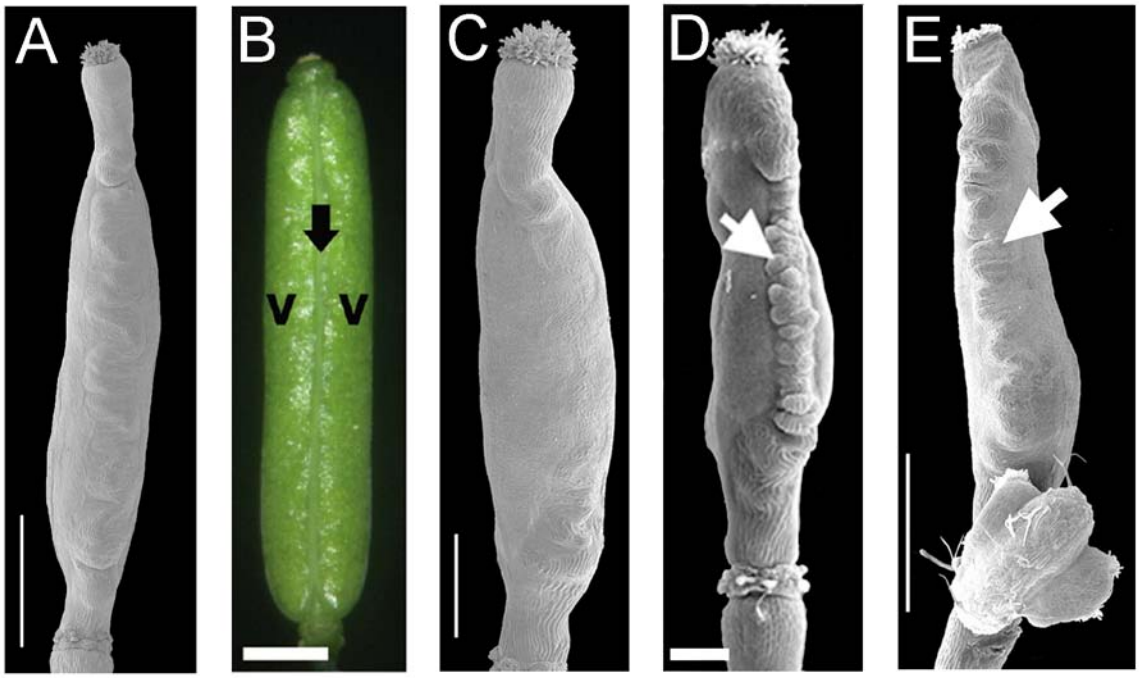


Figure 2. Fruit mutant phenotypes.
(A) SEM of *ful* mutant fruit. **(B)** Whole mount of *rpl* fruit with labeled valve (v), and arrow denoting lack of replum. **(C)** SEM of *ful rpl* double mutant fruit. **(D)** SEM of *ful rpl m33* triple mutant fruit from suppressor screen. Arrow denotes rescued replum development. **(E)** SEM of *ful rpl m413* triple mutant fruit. Arrow denotes rescued replum development.

Scale bar in (A) is indicates 1mm and also applies to (B), (C), and (E). Scale bar in (D) indicates 250µm.

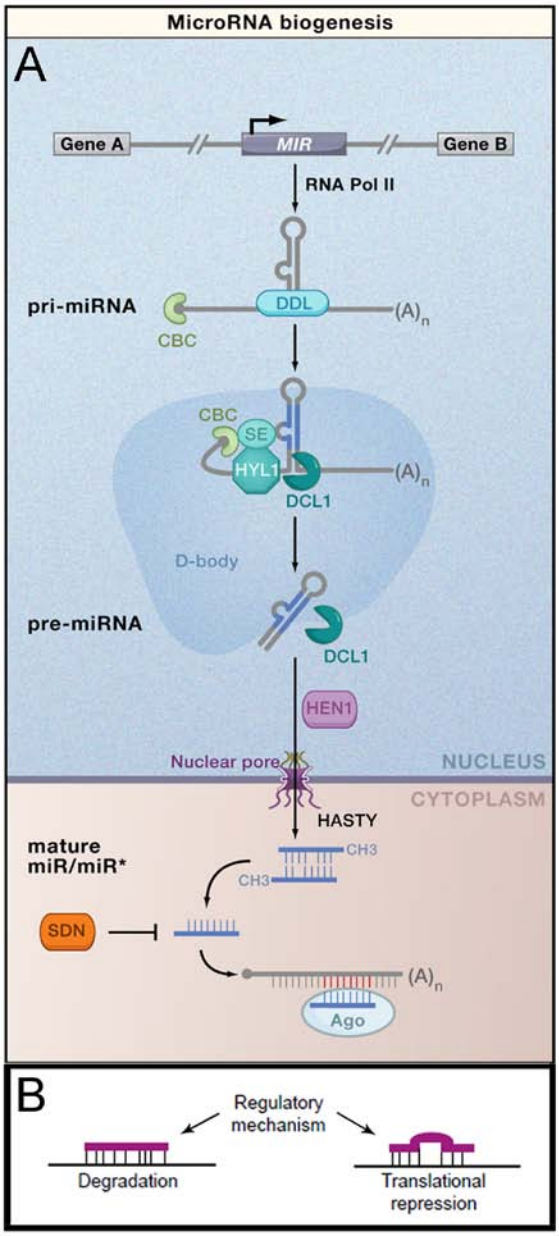


Figure 3. Pathway for microRNA biosynthesis and their regulatory mechanisms. **(A)** microRNAs (miRs) are transcribed by RNA polymerase II (Pol II) in the nucleus from intergenic regions as precursor-miR that then adopt a secondary hairpin structure which subsequently get processed by Dicer-like proteins (DCL1) to form mature dsRNA miRs. HEN1 methylates the duplex to protect the strands from degradation before HASTY exports them to the cytoplasm. One of the strands is degraded and the other (called the guide strand) is loaded onto Argonaute (AGO) proteins in the RNA-induced silencing complex (RISC) in order to carry out its function (Voinnet, 2009). **(B)** AGO enables the activation of the miR so that it can repress gene expression through the degradation of the target mRNA or inhibition of translation of the target mRNA (Pasquinelli et al., 2005).

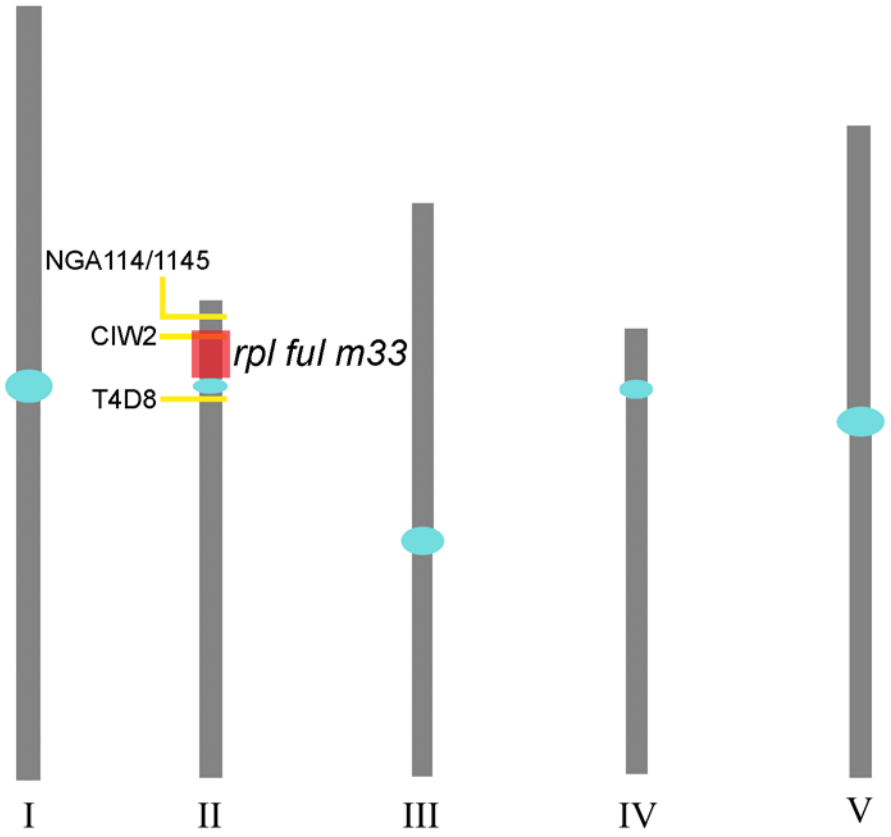


Figure 4. Map of *Arabidopsis thaliana* Chromosomes I-V, showing *m33* is mapped to Chromosome II.
The *rpl ful m33* mutation has been found to be located in the region indicated by the red box at the top of Chromosome II, between the markers NGA114/1145 and T4D8. *m33* was found closely linked to the marker CIW2.

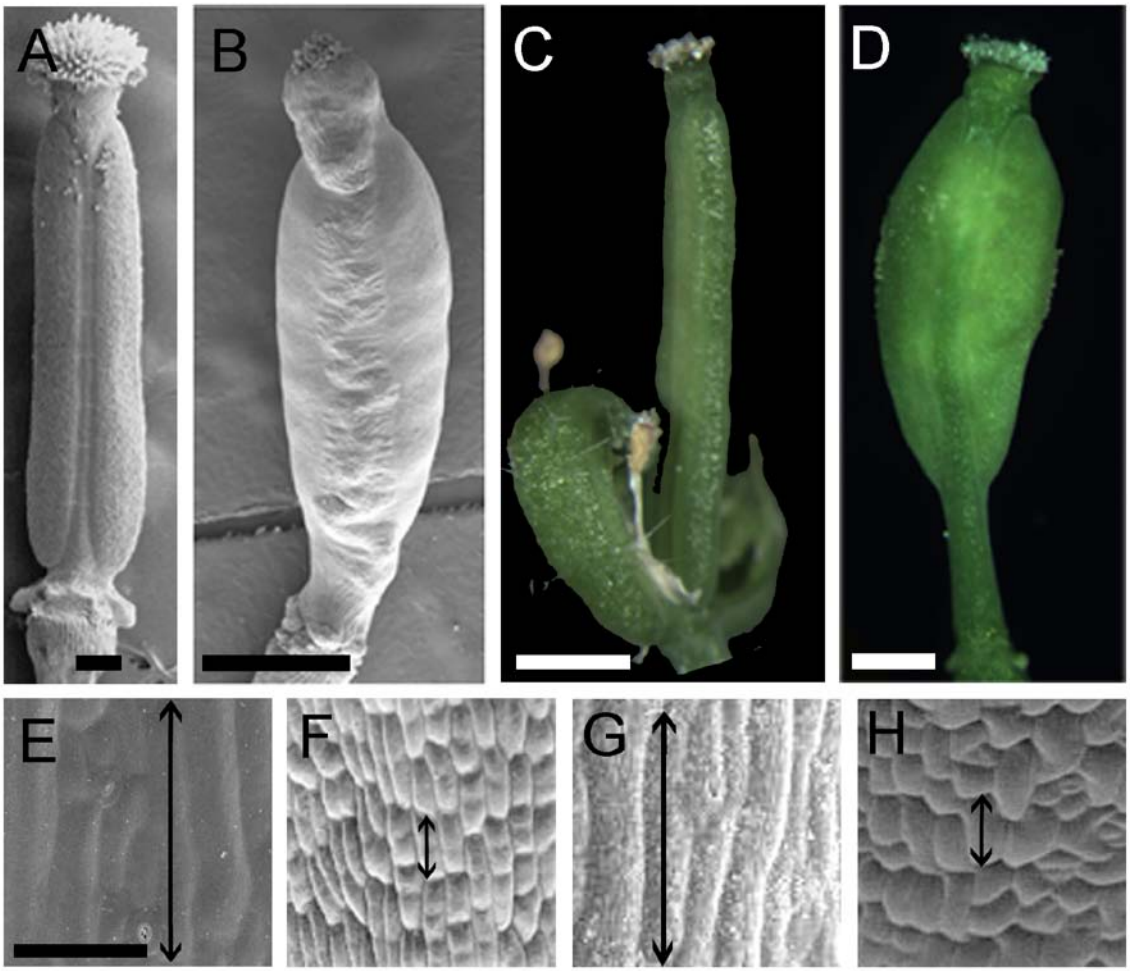


Figure 5. Comparison of cell shapes and sizes between wild-type and mutant fruits from misexpression experiment.
(A) SEM of wild-type fruit gynoecium. **(B)** SEM of *ful* mutant fruit gynoecium. **(C)** Whole mount of *ap2* mutant fruit. **(D)** Whole mount of *FUL>>AP2m* fruit (expression of microRNA-resistant version of *AP2* driven by *FUL* promoter). **(E)** SEM of valve cells of wild-type fruit. **(F)** SEM of valve cells in a *ful-2* mutant fruit. **(G)** SEM of valve cells in an *ap2* mutant fruit. **(H)** SEM of the valve cells in a *FUL>>AP2m* fruit. Double-headed arrows indicate relative cell sizes in each fruit.

Scale bar in (A) indicates 100µm. Scale bars in (B), (C), and (D) indicate 500µm. Scale bar in (E) indicates 50µm and also applies to (F-H).

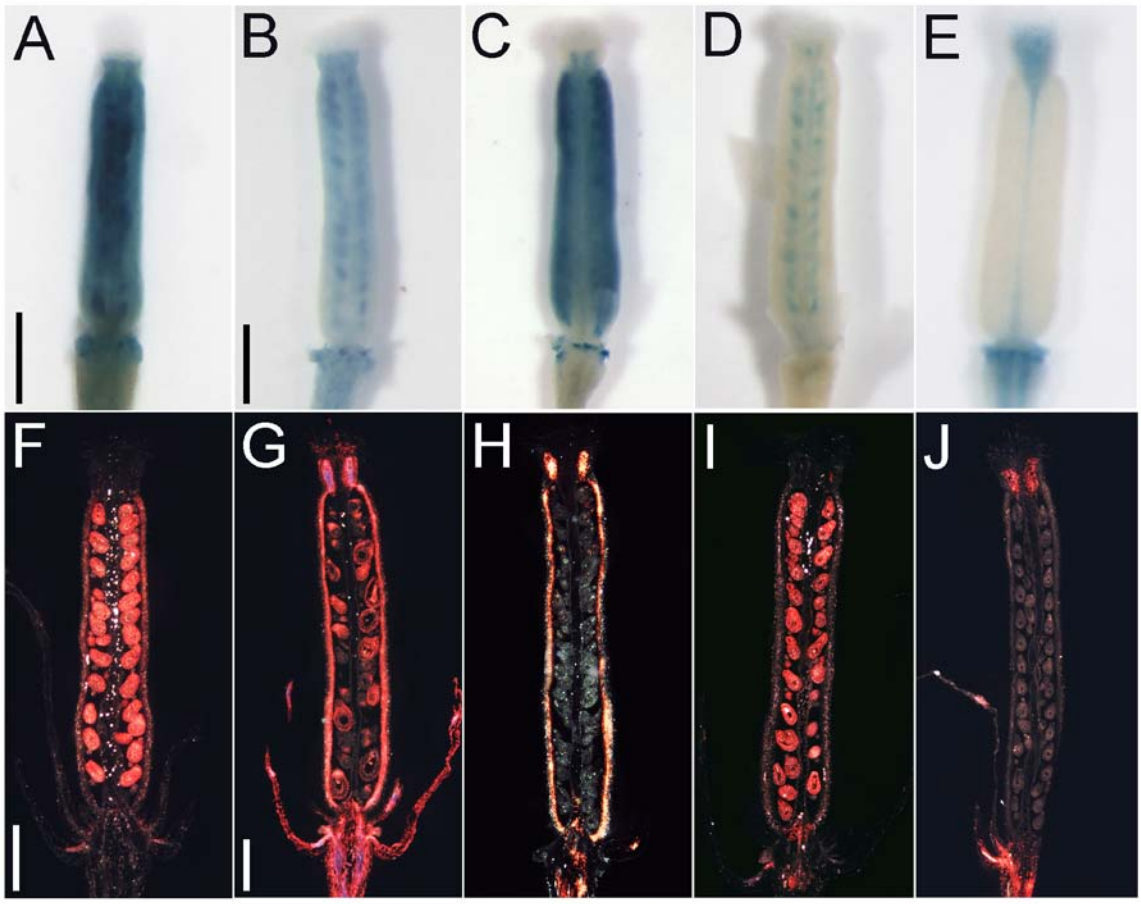


Figure 6. Expression patterns of *miR172::GUS* reporters in fruits of T1 lines (whole mount and longitudinal sections).
GUS expression is shown in whole mount and longitudinal cross-sections for **(A)** and **(F)** *miR172A::GUS* reporter, **(B)** and **(G)** *miR172B::GUS* reporter, **(C)** and **(H)** *miR172C::GUS* reporter, **(D)** and **(I)** *miR172D::GUS* reporter, and **(E)** and **(J)** *miR172E::GUS* reporter. All whole mount fruits are stage 13-14. All longitudinal sections are of fruits in stage 14-15.

Scale bar in (A) indicates 500µm and also applies to (C). Scale bar in (B) indicates 500µm and also applies to (D) and (E). Scale bar in (F) indicates 200µm and also applies to (I). Scale bar in (G) indicates 200µm and also applies to (H) and (J).

Figure 7. Expression patterns of *miR172::GUS* reporters in fruits of T1 lines (cross-sections).
(A-C) Cross-sections for fruits of T1 *miR172A*::*GUS* reporter lines. *GUS* activity for this reporter was consistently seen only in the adaxial layers of the valves and replum, but not the outer layers, and also seen in the ovules at stage **(A)** 11-13, **(B)** 14-15, and **(C)** 16-18. White arrowheads denote that there is no activity in the funiculus. The same pattern of *GUS* expression is seen for the *miR172B::GUS* reporter **(D-F)** in cross sections of fruits at stages **(D)** 11-13, **(E)** 14-15, and **(F)** 16-18, except that at the late stage **(F)**, it is clear that there is no activity in the valve margin (denoted by white arrowheads). Cross sections **(G-I)** show *miR172C::GUS* reporter activity was seen in all layers of the valves in the cross sections of **(G)** stage 11-13 and **(H)** stage 14-15 fruits, but restricted to the inner layers in **(I)** stage 16-18. For the *miR172D::GUS* reporter **(J-L)** activity was consistently seen in the ovules and the inner layers of the replum in all stages **(J)** 11-13, **(K)** 14-15, **(L)** 16-18, but no activity was seen in the outer layers of the replum or the valve margin. Reporter *GUS* activity for *miR172E::GUS* was consistently seen in cross sections of all fruit stages **(M-O)** in the inner replum, but was stronger in the inner valves (denoted by white arrowheads) at **(M)** stage 11-13, decreased by **(N)** stage 14-15, and disappeared by **(O)** stage 16-18. Overall, staining was not seen at the valve margins and the outer layer of the replum for all *miR172::GUS* reporters.

Scale bar in (A) indicates 100µm and also applies to (D), (G), (J), and (M). Scale bar in (B) indicates 100µm and also applies to (E), (H), (K), and (N). Scale bar in (C) indicates 100µm and also applies to (F), (I), (L), and (O).

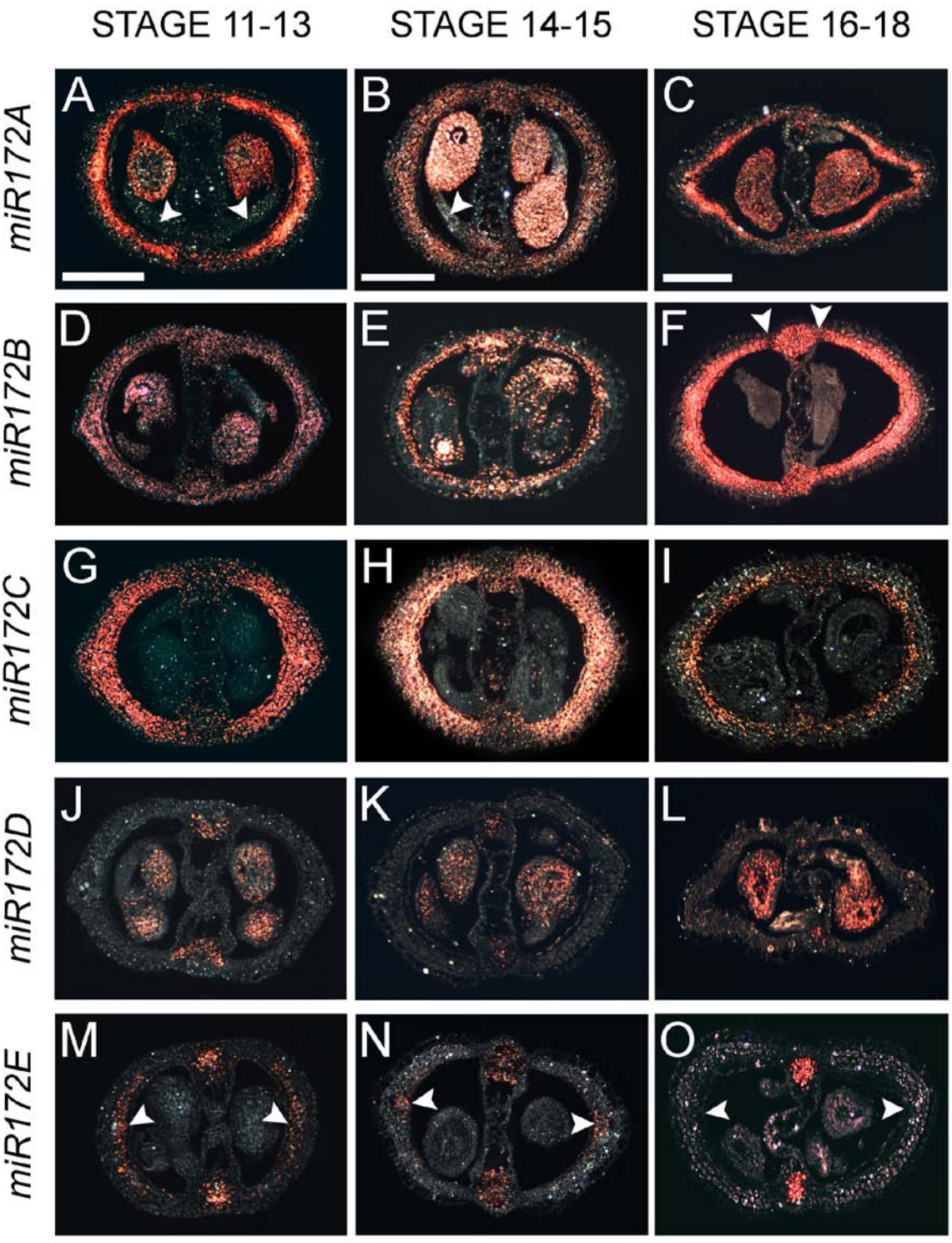
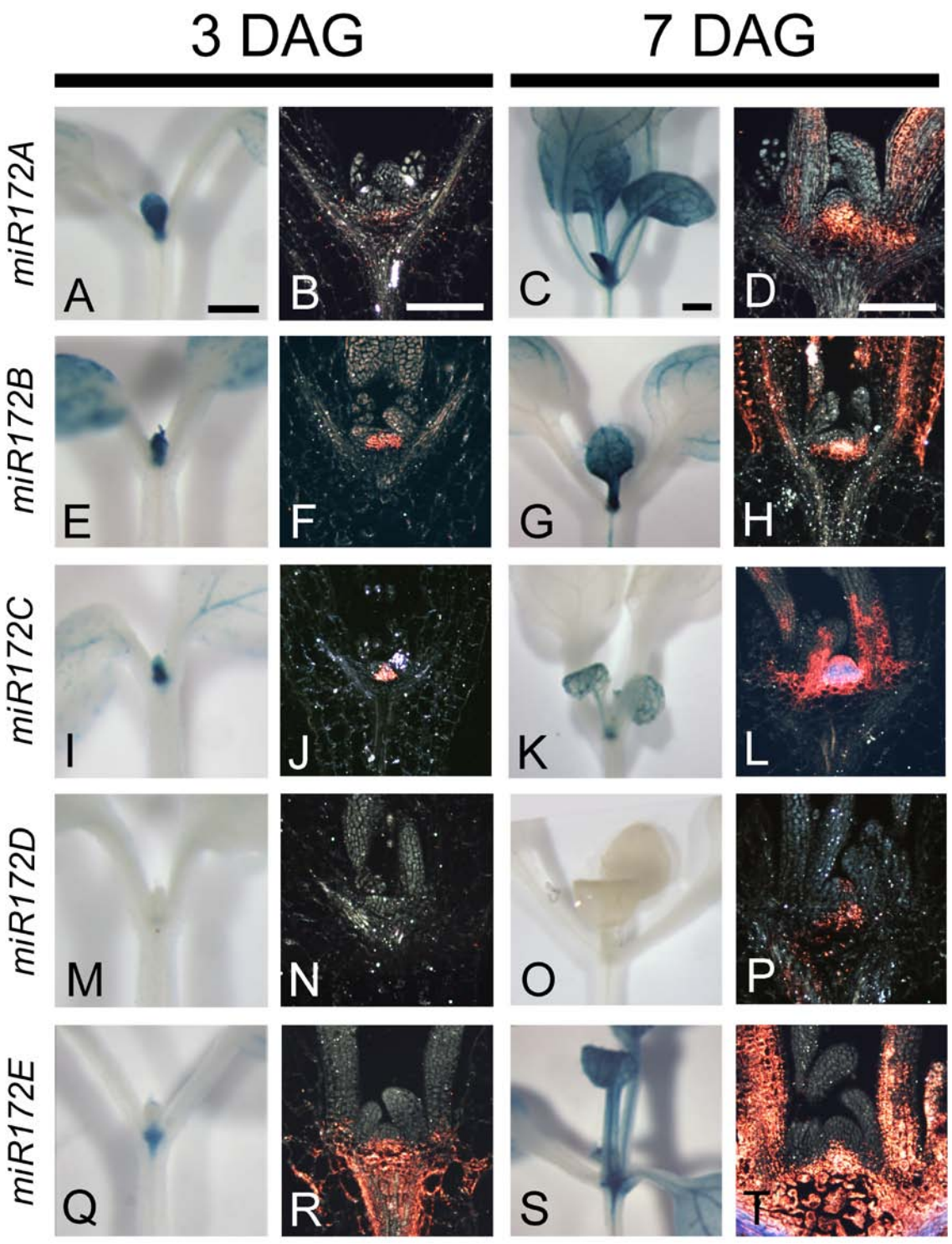

STAGE 11-13
STAGE 14-15
STAGE 16-18
miR172A
miR172B
miR172C
miR172D
miR172E
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 8. Expression patterns of *miR172::GUS* reporters in seedlings.
(A, E, I, M, Q) Whole mounts of seedlings 3 days after germination (3 DAG) for reporter lines **(A)** *miR172A::GUS*, **(E)** *miR172B::GUS*, **(I)** *miR172C::GUS*, **(M)** *miR172D::GUS*, and **(Q)** *miR172E::GUS*. With the exception of **(M)** *miR172D, GUS* activity is seen in the early leaves. **(B, F, J, N, R)** Cross-sections of seedlings showing the shoot apical meristem (SAM) in seedlings 3 DAG for **(B)** *miR172A::GUS*, **(F)** *miR172B::GUS*, **(J)** *miR172C::GUS*, **(N)** *miR172D::GUS*, and **(R)** *miR172E::GUS*. Again, with the exception of the *miR172D::GUS* reporter, *GUS* activity was seen in the regions below the SAM for all *miR172::GUS* reporters. **(C, G, K, O, S)** Whole mounts of seedlings 7 days after germination (7 DAG) for reporter lines **(C)** *miR172A::GUS*, **(G)** *miR172B::GUS*, **(K)** *miR172C::GUS*, **(O)** *miR172D::GUS*, and **(S)** *miR172E::GUS*, where *GUS* activity is seen in the early leaves for all *miR172* reporters except for *miR172D*. **(D, H, L, P, T)** Cross-sections of seedlings showing the shoot apical meristem (SAM) in seedlings 7 DAG for **(D)** *miR172A::GUS*, **(H)** *miR172B::GUS*, **(L)** *miR172C::GUS*, **(P)** *miR172D::GUS*, and **(T)** *miR172E::GUS*. All *miR172::GUS* reporters had activity in the region below the SAM and in the leaves, except for *miR172C::GUS* and *miR172D::GUS*. While **(L)** *miR172C::GUS* reporter activity was seen in the SAM at this later stage, **(P)** *miR172D::GUS* activity was not detected at all in the seedling.

Scale bar in (A) indicates 500µm and also applies to (E), (I), (M), and (Q). Scale bar in (B) indicates 100µm and also applies to (F), (J), (N), and (R). Scale bar in (C) indicates 500µm and also applies to (G), (K), (O), and (S). Scale bar in (D) indicates 500µm and also applies to (H), (L), (P), and (T).


3 DAG
7 DAG
miR172A
miR172B
miR172C
miR172D
miR172E
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

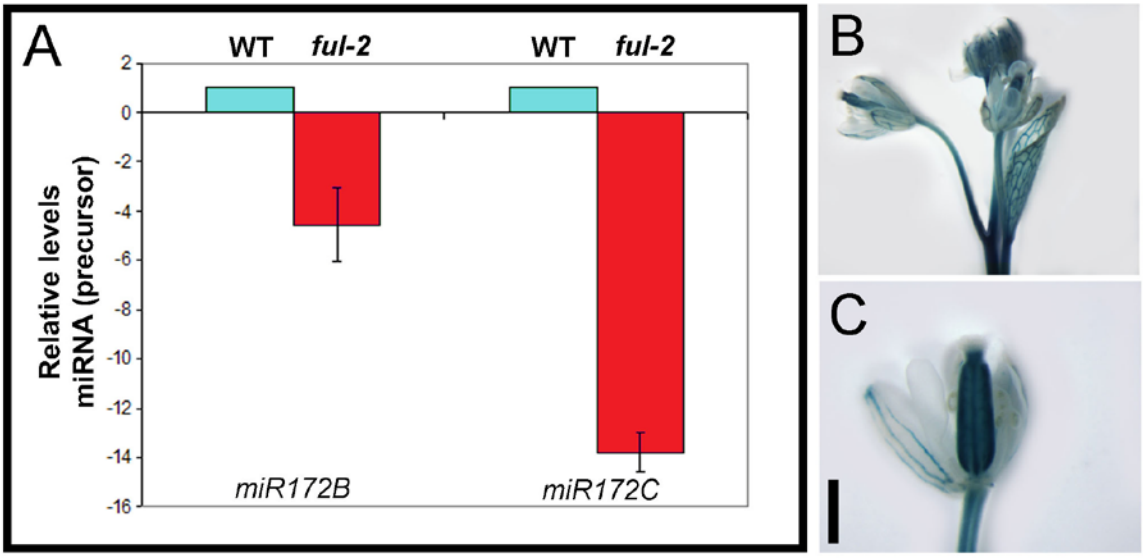


Figure 9. *FUL* positively regulates expression of *miR172B* and *miR172C*.
(A) This graph shows that in the *ful* mutant background, levels of the precursor for both *miR172B* and *miR172C* are down-regulated compared to wild-type background. **(B-C)** From the *35S::FUL* x *miR172C::GUS* cross, overexpression of *FUL* is able to activate transcription of *miR172C::GUS*, so that reporter activity is seen in almost all tissues of the F1 progeny **(B)** inflorescence and **(C)** fruit.

Scale bar in (C) indicates 500µm.

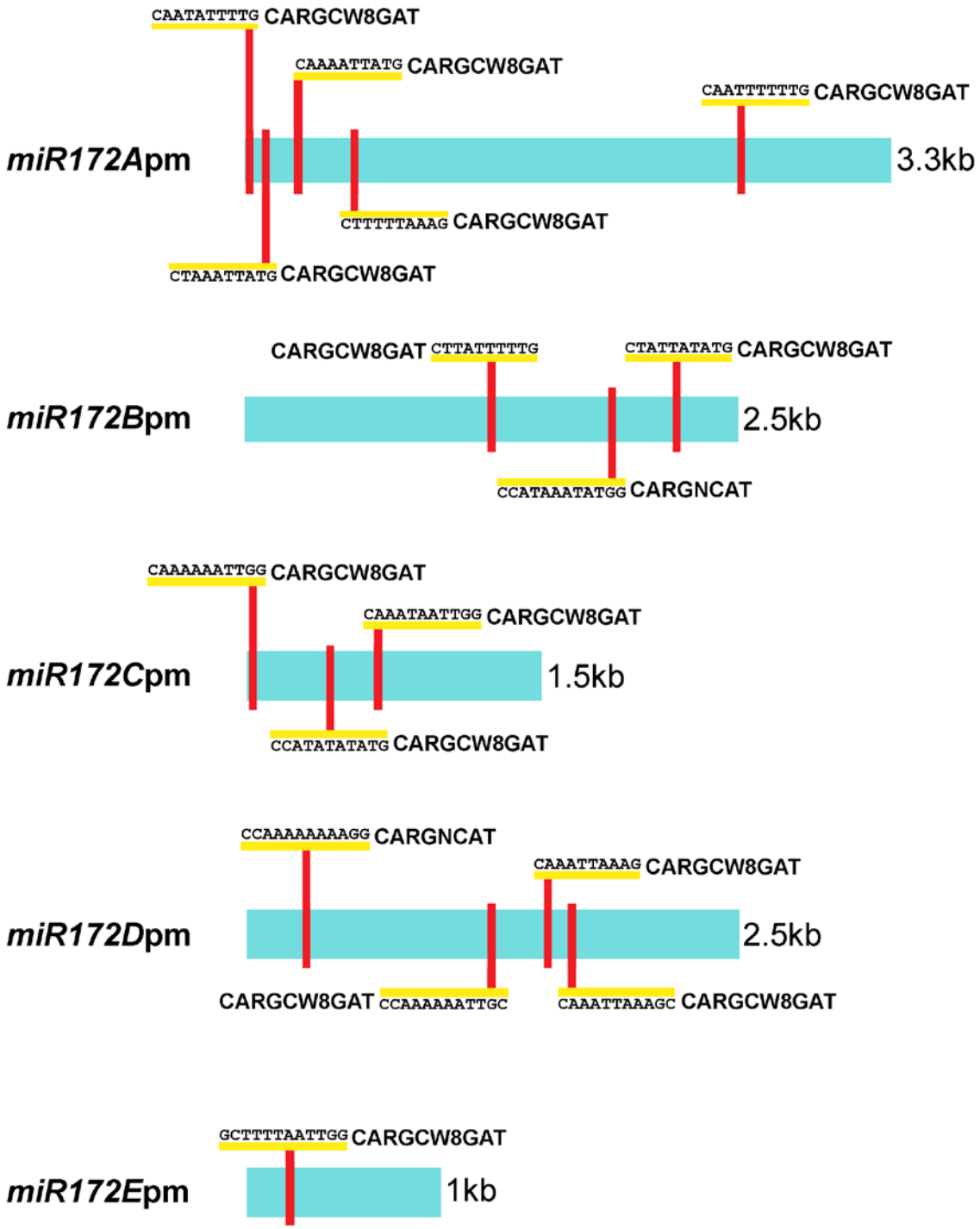


Figure 10. CArG box motifs found in *miR172* promoters.
Diagram of the locations (denoted by red bars) and identities (in capital letters) of CArG boxes found (by using PLACE program at http://www.dna.affrc.go.jp/PLACE/) in the putative promoter regions of *miR172A-E*. Nucleotide sequences of each CArG box is underlined by yellow bar.

APPENDIX B: TABLES AND TABLE LEGENDS

Table 1. SSLP markers on Chromosome II used to map *m33* mutation.
The oligonucleotides (primers) listed were used to amplify SSLP markers in F2 mapping population of *rpl ful* suppressor mutants with rescued replum development to identify linkage for *m33*. Close linkage was found between *m33* and the marker CIW2. These markers are all located on Chromosome II.

R.F. = Recombination Frequency

Marker	Location	Forward Primer (5'—3')	Reverse Primer (5'—3')	R.F. (%)
NGA114	5.7 cM	CCTTCACATC-CAAAACCCAC	GCACATACCC-ACAACCAGAA	65.8
NGA1145	5.7 cM	GCACATACCC-ACAACCAGAA	CCTTCACATC-CAAAACCCAC	65.4
CIW2	9.95 cM	CCCAAAAGTT-AATTATACTGT	CCGGGTTAA-TAATAAATGT	16.4
T4D8	34.0 cM	Y. Zhao Lab	Y. Zhao Lab	69.2
CIW3	53.3 cM	GAAACTCAAT-GAAATCCACTT	TGAACTTGTT-GTGAGCTTTGA	64.9
NGA168	73.8 cM	GAGGACATGT-ATAGGAGCCTCG	TCGTCTACT-GCACTGCCG	79.7
G009	95.5 cM	AACTTACATTC-TTCAATCCTTCG	TGACTAGAGTG-TATTTGATGTGG	78.2

Table 2. Oligonucleotides used to create *GUS* reporter constructs.
The oligonucleotides (primers) listed were used to amplify the putative 5' promoter regions of the *miR172* genes and create restriction sites for cloning into the vector pJJGUS for generating *GUS* reporter lines. Underlined are the restriction sites used for cloning. The 5' primer for *miR172B* does not have a restriction site because there is a KpnI site 44 bp downstream of the primer.

R.E. = Restriction Enzyme

Gene	Length (bp)	5' Primer (5'—3')	3' Primer (5'—3')	5' R.E.	3' R.E.
miRNA172A (At2g28056)	3311	**oJJR167 TTGGTACCAACACGAT-AACAACGAGCAATGAGC**	**oJJR208 TTGGTACCATCCAC-TTCAGACTGTACGGAC**	KpnI	SalI
miRNA172B (At5g04275)	2534	**oJJR171 AAATATATATAATCT-ATGATAATGTGGACAG**	**oJJR172 TTGTCGACCTACAAACAA-CGACAGATGAGCTTTCTTC**	KpnI	SalI
miRNA172C (At3g11435)	1484	**oJJR175 TTGGTACCAACTGCTAT-AGTAGGATCCACATGTGC**	**oJJR176 TTGTCGACGGTTGA-TGATAGGGATGTATG**	KpnI	SalI
miRNA172D (At3g55512)	2545	**oJJR179 TTGGTACCCCTCGATA-GATTTGAGATTCGATAC**	**oJJR180 TTGTCGACGCCACTAAC-TTCAGAATCTGAAGTCATC**	KpnI	SalI
miRNA172E (At5g59505)	1059	**oJJR183 TTGGTACCGTTCAAC-TCTTTGGGTTAGCACG**	**oJJR184 TTCTCGAGTGGCTGA-TAACATCCACCAAAGGC**	KpnI	XhoI

Table 3. Oligonucleotides used for quantitative RT-PCR.
The oligonucleotides (primers) listed were used to detect levels of the miR172 precursors in different mutant backgrounds by quantitative RT-PCR using the LightCycler 3.5 (Roche) system.

Precursor	Primer Name	Primer Sequence (5'—3')
miR172B	oJJR173q	**TTATACAAGTTGTCGGCGGATCCATG**
	oJJR174q	**CGATCCAGACTTCAATCAATATCTTCAAG**
miR172C	oJJR289	**CCGTCTTGAGTCTTGAAAAG**
	oJJR290	**GAAATACCTCCGATCTGTGA**

Table 4. Oligonucleotides for the mutagenesis of CArG boxes of *miR172* promoters. The oligonucleotides (primers) listed were used to mutagenize the CArG boxes in *miR172* promoter regions by PCR. Underlined are the base pair changes made in each CArG box.

Primer Name	Primer Sequence (5'—3')	Original CArG box Sequence (5'—3')	Notes
oQA1	GCAATGGATGATATATATCG-TACATTAGC	CATATATATG	2nd CARGW8GAT in *miR172C*pm
oQA2	AATGTACGATATATATCA-TCCATTGCACCAAAC	CATATATATG	Used with oQA1.
oQA3	CAATGAAGGATAAATATCCT-TCGGTCCGGTTC	CCATAAATATGG	1st CARGNCAT in *miR172B*pm
oQA4	ACCGAAGGATATTTATCCT-TCATTGTCTC	CCATAAATATGG	Used with oQA3.
oQA8	CTTGGGAAAAAAAACCA-ATAAAAAACAAGAAAGAGC	CCAAAAAAAAGG	1st CARGNCAT in *miR172D*pm
oQA9	GTTTTTTATTGGTTTTTTTTCCC-AAGAAATGACTAC	CCAAAAAAAAGG	Used with oQA8.
oQA10	AAGTTGGAAAAAATTCGG-TAGGCAGGATCACAAATGAGAC	CCAAAAAATTGC	2nd CARGNCAT in *miR172D*pm
oQA11	CTGCCTACCGAATTTTTTCCA-ACTTGCTG	CCAAAAAATTGC	Used with oQA10.

Table 5. Expression pattern of each *miR172* reporter in reproductive tissues.
Below is a chart showing the areas of expression in different tissues of the gynoecium for the five *miR172::GUS* reporter lines. Shaded boxes represent positive expression.

	Valves (abaxial)	Valves (adaxial)	Valve Margin	Replum (abaxial)	Replum (adaxial)	Ovules	Style
miR172A							
miR172B							
miR172C							
miR172D							
miR172E							

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