UNVIERSITY OF CALIFORNIA, SAN DIEGO

Combining Classical Approaches and New Technologies to Identify and Explore Novel Regulatory Networks Governing Fruit Development

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

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in

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by

Scott Wu

Committee in charge:

Professor Martin Yanofsky, Chair
Professor Lin Chao
Professor Mark Estelle

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The Thesis of Scott Wu is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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DEDICATION

To my parents, for always encouraging me to be better than who I was yesterday, for never giving up on me, for putting me through college, and supporting me in following my dreams.

To my friends and family, for your words of encouragement, advice, support, and for believing in me when I needed it the most.

To new friends, for the laughs we shared and new memories formed.

EPIGRAPH

"Agriculture is our wisest pursuit, because it will in the end contribute most to real wealth, good morals, and happiness."

Thomas Jefferson, Letter to George Washington, 1787

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ABSTRACT OF THE THESIS

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Professor Martin Yanofsky, Chair

Arabidopsis fruit are patterned into three major regions: the valves, replum, and valve margin. Previous studies have shown that different suites of transcriptional regulators control the formation of each of these tissue types and modulate their growth. However, our recent findings have revealed the significant impact that microRNA (miR)-guided post-transcriptional control has during fruit development. Our goal in this study is to incorporate the activities of these miRNAs and their corresponding targets into the current regulatory networks governing fruit development. At the same time, we have also developed several strategies to elucidate the upstream layer of transcriptional regulators modulating the activity of these riboregulators.

Through the combination of traditional approaches, high-throughput technologies, and bioinformatics tools, we have identified several putative upstream regulators of two miRNA

encoding loci that play a role in fruit development. Although we have only scratched the surface of understanding the regulatory networks orchestrating this process, our research has opened the door to more efficient methods of identifying key regulators in the fruit development pathway.

INTRODUCTION

We are constantly reminded of the wise words given by Norman Borlaug in his 1970 Nobel Lecture, "Civilization as it is known today could not have evolved, nor can it survive, without an adequate food supply." Borlaug, the father of the green revolution, is often credited for saving millions from starvation by creating high-yield crops and modernizing agricultural management techniques via a series of research and development. Today, as the world's population continues to skyrocket, we are constantly looking for ways to feed and sustain the world population.

In recent years many studies haven been initiated to analyze fruit development. Fruits are the harvested product of many crop species and have an important impact on diet and economy. While the general populous is familiar with the nutritional benefits of eating fruit, the other benefits and uses of fruit are often overlooked. Much of modern medicine and pharmaceutical practices rely on key vitamins, minerals, and extracts found only in fruits. Furthermore, oils contained within fruits of plants such as *Jatropha curcas* can be used for alternative fuel sources (Worapun et al., 2012). With such a wide range of uses, an understanding of fruit development could help researchers control or manipulate traits to increase quantity and quality of fruit crop species to improve their agricultural importance.

The model organism, *Arabidopsis thaliana*

Arabidopsis thaliana (Arabidopsis hereafter*)* belongs to the Brassicaceae family. This small flowering plant is closely related to cabbage and mustard. *Arabidopsis* is of great interest and value in the plant biology world because it grows quickly, phenotypic changes are very easily observed, and it was the first plant to have its entire genome sequenced. The mature plant is small in size making it easy to store and grow and contains many characteristics, such as life cycle, fruit development, and genomic organization, which are comparable to other

plant species. Additionally, it has been found that many *Arabidopsis* genes have close relatives (orthologs) in many other plant species, such as tomato, peach, and cherry (Kitashiba et al., 2004; Dardick et al., 2010; Shindo et al., 2012). Lastly, and perhaps what makes *Arabidopsis* so useful in a research environment is the ease in which new genetic information can be introduced and expressed via *Agrobacterium tumefacians* transformations (Clough and Bent, 1998; Meyerowitz, 1989).

Arabidopsis thaliana fruit structure

The reproductive organs of *Arabidopsis* develop after the transformation of the shoot apical meristem into inflorescence meristems. *Arabidopsis* flowers, each containing four whorls of organs, develop laterally from each inflorescence meristem (Figure 1A). Four sepals make up the outermost whorl, followed by four petals, which surround a ring of six stamens (male reproductive organs), and lastly two carpels that are fused together forming the gynoecium (female reproductive organ; Figure 1B) (Dinneny and Yanofsky, 2005). Fruit development begins once fertilization occurs.

Arabidopsis fruit takes form in the shape of a long cylindrical structure known as the silique, in which the gynoecium is located. The gynoecium is composed of the ovary and the stigma, a layer of cells where pollen attaches and fertilizes the plant (Figure 1B) (Bowman et al., 1999; Crawford and Yanofsky, 2008). Inside the ovary are the ovules, which develop into seeds upon fertilization.

The outer walls of the ovary can be further split into three regions – valves, valve margins, and replum. The primary function of valve tissue, forming the lateral walls of the ovary (Figure 1C), is to protect the developing seeds within the ovary. Valve margins, which are comprised of a lignified layer (on the valve side) and separation layer (on the replum side), connect the valves to the replum (Figure 1C). Also known as the dehiscence zones, enzymatic

processes within the valve margins degrade the separation layer, separating the two layers, while the lignified layer creates tension causing a detachment of the valves from the replum, releasing the seeds contained within the ovary (Spence et al., 1996; Liljegren et al., 2000; Dinneny and Yanofsky, 2005; Arnaud et al.). The replum lies in the medial region of the fruit and shows meristematic activities necessary for the formation of inner structures (Figure 1C).

Genetic network regulating fruit morphogenesis

Utilizing *Arabidopsis* fruit as a platform, the studies performed in the laboratory of Prof. Martin F. Yanofsky have proven pivotal in uncovering the regulatory genes controlling the formation of this organ. It has been discovered that genes regulating valve, valve margins and replum identities negatively regulate one another to maintain the fate of their specific domains of expression, which allows proper fruit morphogenesis (Figure 2).

The main regulatory gene active in valve tissue has been discovered to be the MADS-box domain transcription factor gene *FRUITFULL (FUL)* (Gu et al., 1998). In *ful* mutant fruits, severe reduction in valve cell length is observed along with expansion of the replum (Gu et al., 1998; Liljegren et al., 2004). In fruits where *FUL* is over-expressed, cells with valve identity cover the entire ovary surface, indicating that *FUL* plays a critical role in regulating proper valve formation. Additionally, in the valves, *FUL* negatively regulates the valve margin genes *SHATTERPROOF1 (SHP1)*, *SHATTERPROOF2 (SHP2)*, *ALCATRAZ (ALC),* and *INDEHISCENT (IND)* (Liljegren et al., 2000; Rajani and Sundaresan, 2001; Roeder et al., 2003; Liljegren et al., 2004; Dinneny et al., 2005).

Within the valve margins, *SHP1, SHP2, ALC,* and *IND* play roles in determining cell fate between the two layers. *IND, SHP1,* and *SHP2* have been found to play an important role in both separation and lignified layers (Ferrandiz et al., 2000a; Liljegren et al., 2000; Pinyopich et al., 2003; Favaro et al., 2003; Liljegren et al., 2004; Roeder et al., 2005). Mutant *shp1,2* fruits lack

defined valve margin areas and while mature fruit do form, they cannot dehisce. The same phenotype is also observed in *ind* mutants. However, unlike *SHP1, SHP2,* and *IND, ALC* is expressed only in the separation layer (Rajani and Sundaresan, 2001). Two other genes discovered to be expressed in valve margins are *KNOTTED-LIKE HOMEOBOX GENE 2 (KNAT2)* and *KNOTTED-LIKE HOMEOBOX GENE 6 (KNAT6)* (Ragni et al., 2008). These two genes seem to prevent the expression of the replum identity genes in valve margin tissues (Venglat et al., 2002; Byrne et al., 2003; Bhatt et al., 2004; Ragni et al., 2008).

The class I KNOX gene *BREVIPEDICELLUS* (*BP*), regulates replum growth by closely cooperating at the molecular level with the BELL1 homeodomain transcription factor *REPLUMLESS (RPL)* (Ferrandiz et al., 1999; Robles and Pelaz, 2005; Roeder et al., 2005; Arnaud et al.; Ripoll et al., 2011). In *rpl* mutants, replum size is drastically reduced and is replaced with valve margin cells. In *rpl bp* double mutants, the fruit contains no replum, indicating that *RPL* and *BP* are required for replum development.

Interestingly, FUL and RPL prevent the ectopic expression of the valve margin identity gene in the valves and in the replum, respectively. The *ful rpl* double mutant shows ectopic expression of valve margin cells and the disappearance of valves and replum.

Recent research has uncovered a few additional regulatory genes involved in fruit development: *APETALA 2 (AP2), ASYMETRIC LEAVES 1 (AS1), ASYMETRIC LEAVES 2 (AS2), FILAMENTOUS FLOWER (FIL), YABBY 3 (YAB3),* and *JAGGED (JAG)* (Sawa et al., 1999; Siegfried et al., 1999; Roeder et al., 2003; Dinneny et al., 2005; Alonso-Cantabrana et al., 2007; Ikezaki et al., 2010; Li et al., 2012). *AP2*, a member of the A function of the ABC model for flower development, has been reported before to be post-transcriptionally regulated by microRNA172 (miR172) (Aukerman, 2003; Chen, 2004). During fruit development, *AP2* negatively regulates replum and valve margin growth by repressing the replum and the valve margin identity genes, respectively. However no role for AP2 has been found in valves so far

(Mathieu et al., 2009; Wollmann et al., 2010; Yant et al., 2010; Ripoll et al., 2011). *AS1* and *AS2* seem to play a role in negatively regulating class I KNOX genes, which include *BP* in replum tissue and *KNAT2* and *KNAT6* in valve margin tissue (Alonso-Cantabrana et al., 2007; Guo et al., 2008; Ragni et al., 2008). In addition, *AS1* and *AS2* collaborate with *FIL, JAG,* and *YAB3* in allowing *FUL* expression in valve tissue.

MicroRNAs (miRs) and plant development

About a decade ago, the discovery of microRNAs (miRs) completely changed the understanding of how genes are regulated. MicroRNAs, short 21-24 nucleotide non-coding RNA sequences, modulate the activity and function of their target genes via translational repression or target degradation (Figure 6A) (Hake, 2003; Pasquinelli et al., 2005; Garcia, 2008; Filipowicz et al., 2008; Carthew and Sontheimer, 2009; Voinnet, 2009; Colaiacovo et al., 2012; Powell and Lenhard, 2012). It has now been found that miRs play a role in almost all aspects of plant development, from root and meristem growth to embryonic development (Figure 3) (Sunkar, 2007; Rubio-Somoza et al., 2009; Colaiacovo et al., 2012; Powell and Lenhard, 2012). With miRs playing such a crucial role in regulating gene expression, it is also not surprising that miRs are also tightly regulated (Krol et al., 2010). The new model now incorporates transcription factors regulating miRs, which in turn regulate other transcription factors, creating a feedback loop of regulation between miRs and transcription factors (Figure 4A).

Using classical and high-throughput approaches to understand biological processes

Advances in molecular biology, bioinformatics and biotechnology have given us a wide arsenal of techniques to study mutations and the role/s of specific genes in a particular biological process. The use of high-throughput techniques is rapidly increasing in virtually all the research fields. At the same time, scientists have generated algorithms and mathematical approaches to

generate methods for standardizing and comparing the data to generate databases within the public domains to aid the scientific community.

In recent years, the combined use of multiple strategies to dissect how a particular process is synthesized is referred to as systems biology. Following these strategies has led to the uncovering of co-regulation networks and immune-associated genes in *Arabidopsis* (Liu et al., 2008; Atias et al., 2009; Liu et al., 2012), stress responsive gene expression in sugarcane leaves (Patade et al., 2012), gene regulatory pathways in rice via the use of the ATTED-II program (Obayashi et al., 2011), specific pathways involved in cacao resistance and susceptibility to witches' broom disease (da Hora Junior et al., 2012), and the role of microRNAs with respect to neurofibromatosis (Lee et al., 2012).

One example of a database developed to aid system biologists is Genevestigator (Hruz et al., 2008). Genevestigator is a reference expression database that documents gene expression across a wide variety of organisms, including *Arabidopsis*. Subsequent revisions of Genevestigator incorporate research data from laboratories around the world to analyze gene expression patterns during different stages of development, responses to different stimuli, and in different tissues. Another database that has been developed is PLACE, a library containing motifs found in plant cis-acting regulatory DNA elements, allowing researchers to see if specific gene sequences contain cis-regulatory motifs (Higo et al., 1999).

Whereas the Yanofsky group has already found a plethora of transcriptional regulators controlling fruit development, our recent research has shown that small RNAs also play a critical role in this process. Our lab is currently investigating the role of miR159, miR167, miR172, and miR390, among others, during fruit development. The target of miR159 has been found to be the two GAMYB-like genes *MYB33* and *MYB65* (Alonso-Peral et al., 2010; 2012), miR167 targets are the *AUXIN RESPONDING FACTOR 6 (ARF6)* and *AUXIN RESPONDING FACTOR 8 (ARF8)* (Nagpal et al., 2005; Ru et al., 2006; Wu et al., 2006), miR172 has been found to target

AP2 and its closest relatives (Aukerman, 2003; Chen, 2004) and the targets of miR390 is the *TAS3* messenger (Marin et al., 2010). This target recognition leads to the formations of the TAS3-derived trans-acting short-interfering RNAs (tasiRNAs), which have been found to inhibit *AUXIN RESPONDING FACTOR 3 (ARF3)* and *AUXIN RESPONDING FACTOR 4 (ARF4)* (Felippes and Weigel, 2009; Marin et al., 2010; our unpublished data). To better understand their roles and how they are regulated, we utilized classical biological approaches (ie mutagenesis and expression analysis) and systems biology tools (ie promoter hiking and bioinformatics analysis).

Two of the techniques and technologies used prominently throughout this study were the microRNA target mimicry and yeast one-hybrid screens. MicroRNA target mimicry technology (Todesco et al., 2010) uses an artificial non-cleavable miR target (MIM) capable of decaying the activity of a specific miR *in vivo* by mimicking the miR target and preventing translational repression or target degradation (Franco-Zorrilla et al., 2007; Chitwood and Timmermans, 2007; Eamens and Wang, 2011; Rubio-Somoza and Manavella, 2011). Because we wanted to observe the effects of lowering a particular miR in a tissue-specific fashion, we combined the MIM technology with the LhGAL4 two component system (Moore et al., 2006), allowing us to express each MIM in specific fruit territories using the corresponding driver line. Utilizing this technology allowed us to observe the effects of lowering specific miR levels in specific plant tissue.

To uncover the upstream transcriptional regulatory network governing the expression of miR-encoding genes, we used the yeast one-hybrid (Y1H) system. Y1H is a system used to identify binding peptides, in this case, transcription factors, that interact with a specific DNA sequence of interest (Dey et al., 2012). Y1H works by using the DNA sequence as bait and assaying its interaction with transcription factors tagged with an activation domain. If the transcription factor binds, the activation domain recruits RNA polymerase and transcription of the promoter and a downstream reporter gene, in this case, the LacZ reporter, is initiated and the

levels of reporter can be measured. In collaboration with Prof. José Pruneda-Paz, we screened our *MIR* promoter sequences, using the Y1H system, with a transcription factor library generated in the Pruneda-Paz lab.

The data obtained from our studies demonstrates that combining both classical and high-throughput approaches can be used to more efficiently identify putative regulators in the regulatory network controlling fruit development.

MATERIALS AND METHODS

All plant work in this study was performed in the *Arabidopsis thaliana* Columbia (Col) accession.

Site-directed mutagenesis

The two auxin response element (AuxRE) motifs (Fig. 10A) contained within the 1.5 kb *MIR172C* promoter were mutagenized to create a derivative of the *MIR172C::GUS* reporter (Fig. 6). The *MIR172C::GUS*$^{\text{AuxRE -/-}}$ (both AuxRE sites mutated) construct was made by generating two fragments with each fragment containing both AuxRE mutations. The first fragment was generated using oJJR175 and oJJR398 primers, and the second fragment was generated using oJJR176 and oJJR398 primers. The two fragments were then combined and the full-length sequence was generated using the oJJR175 and oJJR176 primers. All oligonucleotides and restriction enzymes used to create and verify these constructs are included in Table 1, with the mutated sequences indicated.

To create the transcriptional *β-glucuronidase* (*GUS*) *MIR172C*$^{AuxRE-/-}$ reporter, the *MIR172C*$^{AuxRE-/-}$ promoter generated previously was amplified and isolated using the proof-reading, high-fidelity Taq Polymerase (Phusion from New England Biolabs). The PCR product was subsequently digested by *Kpn*I and *Sal*I and inserted into the pJJGUS vector digested with the same enzymes (Ripoll et al., 2006). After insertion, the construct was transformed into *Escherichia coli*. Joint integrity in the construct was checked by sequencing.

Cloning strategy and transgenic plants

To create the MIMICRY lines, we amplified the *MIM172* and *MIM159* clones created in the Weigel lab (Todesco et al., 2010) using Taq Phusion and the oJJR267 and oJJR268 primers

(Table 2). The resulting PCR product was digested with *Kpn*I and *BamH*I (Table 2) and inserted into the pBJ10xOP vector. After insertion, the 10xOPMIM cassette was excised with *Not*I and inserted into the pGreenII0179 vector. After insertion into the pGreenII0179 vector, the construct was transformed into *Escherichia coli.* Joint integrity in the construct was checked by sequencing.

All of the generated constructs mentioned above, along with the pSOUP helper plasmid (Hellens et al., 2000), were transformed to *Agrobacterium tumefaciens* (AGL0 strain) by electroporation. For plant transformations we followed the floral dip method (Clough and Bent, 1998). T1 transgenic plants harboring the corresponding *GUS* reporter were isolated by sowing seeds on MS plates containing 20 mg/ml Hygromicin.

GUS staining

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

Yeast strains and medium

Yeast strain YM4271 and Mav103 were used to harbor the bait DNA and Gal4-AD-TF strains respectively. The components of yeast complete medium (YPAD) and the different synthetic drop-out (SD) media were purchased from BP and MPBio, respectively, and prepared according to the manufacturer's instructions.

DNA assembly and subsequent transformation into yeast

The oJJR175 and oJJR176 primers were used for the whole *MIR172C* (AT3G11435) promoter region (Table 1) and amplified using Phusion Taq polymerase followed by gel isolation. Each fragment was then ligated into the pLacZi vector from Clontech. Each pLacZi promoter fragment plasmid was then linearized with *Nco*I and transformed into yeast strain YM4271. 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 OD600 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 re-suspended in 1.5ml of fresh 1xTE (10 mM Tris pH 7.5, 0.05 mM EDTA) /1xLiAc (pH 7.5). 100µl of competent yeast was then added to 10µl of digested plasmid and 10µg of single stranded carrier salmon sperm DNA. 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. 70µl of DMSO was added to each transformation and mixed by gentle inversion. Cultures were then heat shocked at 42°C for 15min and then iced for 2 minutes before being centrifuged for 10 seconds at max speed. The supernatant was removed, re-suspended in 150µl 1xTE, and plated on SD –ura and incubated at 30°C in a humidified box for 3 days. Colonies were then checked by PCR with original amplification primers and pLacZi forward and reverse primers.

Yeast-1-hybrid

The yeast-1-hybrid assays were performed by Jose Pruneda-Paz as previously described (Pruneda-Paz et al., 2009).

Transient Expression Assay

The transient expression in tobacco leaves was performed using a modified protocol (Guan et al., unpublished).

Cytoscape and Genevestigator Analysis

Data from the yeast 1-hybrid assays were imported into excel and sorted by fold of induction. A series of filters were applied to the data (described in the discussion section), and putative candidates identified after filtering. Putative candidates were then imported into Cytoscape and organized using the cytoscape force-directed layout, using fold of induction as the filtering factor (Smoot et al., 2011). A custom visual map scheme was designed to distinguish between transcription factors and miRNAs and to display if they were putative negative regulators (Figures 14-15; 18-19; 23). Top putative candidates were then imported into Genevestigator in groups of five (Figures 16-17; 20-22) using the "developmental expression" tool and the Arabidopsis AT-22K array (Hruz et al., 2008).

RESULTS

FUL regulation of *MIR172C* in valve tissue represses *AP2*

As mentioned previously, *AP2* has been shown to negatively regulate growth in the replum and valve margin regions during fruit development. Therefore, in *ap2* mutant fruits replum and valve margin regions were clearly enlarged. Valves and valve tissue cells remain normal and no changes in size or shape were detected. However, while *AP2* messenger was detected in valve tissue, AP2 protein was largely absent (Ripoll et al., 2011), indicating that something was preventing *AP2* messenger to be translated in valve tissue.

AP2 (as well as its closest relatives) has been shown to be post-transcriptional regulated by a small riboregulator called miR172 during flower development and flowering time (Aukerman, 2003; Chen, 2004; Mathieu et al., 2009; Wollmann et al., 2010; Yant et al., 2010). To investigate the effects of miR172 in valve development, we decided to take advantage of target MIMICRY technology (Todesco et al., 2010). This strategy uses an artificial non-cleavable microRNA target (MIM) capable of decaying a specific microRNA activity *in vivo* (Franco-Zorrilla et al., 2007; Chitwood et al., 2007; Eamens and Wang, 2011; Rubio-Somoza and Weigel, 2011). A MIM-targeting miR172 (*MIM172*) construct was generated and mis-expressed in plants using the CaMV35S promoter. As shown in Figure 7B, the overall length of *35S::MIM172* fruit was smaller and the size of epidermal valve cells was reduced (not shown). Additionally, we generated a miR172-immune version of *AP2* cDNA and expressed it in fruit valves using the *FUL* promoter. Fruits from these plants also show a drastic reduction in valve length, closely resembling the phenotype of *ful* mutants (Figure 7E). This data strongly suggests that miR172 is likely repressing AP2 function in valve tissue, much like it does during flower development and flowering time. This was confirmed by studying the expression pattern of miR172-conding genes. Strikingly, *MIR172C::GUS* was active throughout the valves (Mai, 2009) overlapping with the

known expression patterns of *FUL*. It was later found that *FUL* regulates *MIR172C* directly through at least one of the CarG-boxes (cis-regulatory motifs for MADS-box transcription factors) found on the *MIR172C* promoter (our unpublished data). However, although this regulation is important, *MIR172C::GUS* reporter was still found to be active in *ful* mutants, suggesting that additional regulatory genes participate in its regulation.

***MIR172C* promoter contains two active canonical AuxRE cis-regulatory motifs**

Besides the CArG-boxes, in silico analysis using PLACE (Higo et al., 1999) and PlantPAN web-based platforms (Chang et al., 2008) revealed the presence of additional cis-regulatory motifs, including two Auxin Responding Elements (AuxREs) downstream of the third CarG box motifs mentioned earlier (Figure 9A). AuxREs are the DNA targets of regulatory sequences recognized by AUXIN RESPONSE FACTORS (ARFs) that can either repress or activate target gene expression (Ulmasov et al., 1999; 1997; Dharmasiri and Estelle, 2002; Cole et al., 2009).

We decided to first test the functionality of these cis-motifs and generated a mutated version of *MIR172C* promoter in which both AUXREs were mutated (TGTCTC→ ATATAT). As a preliminary test, we conducted a TAT (Transient Assay in Tobacco) assay, via *Agrobacterium* leaf infiltration, in which the activity of $MIR172C^{AuxRE-/-}$*::GUS* was assayed and compared to that of $MIR172C^{WT}$*::GUS*. While $MIR172C^{WT}$*::GUS* was active throughout the control leaf, only small amounts of *GUS*-signal were observed in $MIR172C^{AuxRE-/-}$*::GUS* infected leaves. These results strongly suggest that both AuxRE sites are required for *MIR172C* expression.

MIR172C$^{AuxRE-/-}$ expression patterns in reproductive tissues

Our next step was to generate *Arabidopsis* transgenic lines harboring *MIR172C*$^{AuxRE-/-}$*::GUS* and assay GUS expression in fruits. For *MIR172C*$^{AuxRE-/-}$*::GUS*, twenty-five T1 lines were generated. *GUS* activity was checked in reproductive (pistils and fruits) tissue during pre- and post-fertilization stages. *GUS* activity was also checked in vegetative (roots) tissue once the plants had bolted (unpublished data). *GUS* expression patterns in T1 lines were corroborated in T2 lines. Expression patterns were consistent throughout the majority of the lines. Whereas *MIR172CWT::GUS* fruits showed high levels of expression throughout the entire valve, fruit in *MIR172C*$^{AuxRE-/-}$*::GUS* showed a drastic reduction in the overall levels of expression and only residual signal was detected in the top (and in the bottom in some fruits tested) part of the valves (Figure 10C). Like the TAT assay, this data suggests a positive role for both AuxREs in the control of *MIR172C* expression.

ARF6,8 are the best candidates for regulating *MIR172C* in fruit valves

Knowing that ARF transcription factors are involved in regulating *MIR172C,* we decided to take a look at the ARF6 and ARF8 transcription factors first, as they have been found to play a role in floral organ development and flower maturation (Nagpal et al., 2005; Wurschum et al., 2006; Tabata et al., 2010). A comparison of wild type, *arf6 -/- arf8 +/-* single mutant*,* and *arf6 arf 8* double mutant fruits reveal the *arf6 arf 8* double mutant fruit closely resemble that of ectopically expressed *MIM172* while the *arf6 -/- arf8 +/-* sesquimutant takes on an intermediate phenotype, suggesting both ARFs are needed for normal *MIR172C* regulation.

Therefore, we tested the activity of *MIR172C::GUS* in *arf6*,8 mutant combinations. In the *arf6 -/- arf8 +/-* sesquimutant fruits, there was some residual expression in valve tissue whereas in *arf6 -/- arf8 -/-* double mutants, no expression of *MIR172C::GUS* was found or at least not detected using our GUS-assay protocol (Figure 9C).

All together, our data indicates that the AuxRE sites located on the *MIR172C* promoter play an important role in proper miRNA expression, particularly in the central region of the fruit. This is not surprising since *ful* fruits show *MIR172C* expression in the same region (unpublished data). It can be summarized that ARF6 and ARF8 play a key role in proper *MIR172C* expression in valve tissue, based on the phenotypes of *arf6* and *arf 8* mutants as well as the *GUS* expression patterns. However, further experiments need to be conducted to determine if *MIR172C* is directly regulated by ARF6 and/or ARF8. We are currently generating constructs to perform a ChIP assay to prove direct regulation of *MIR172C* by ARF6 and ARF8.

miR159 likely participates in fruit growth

To identify and explore more miR-modulated regulatory circuits, we decided to generate MIMICRY constructs to explore two additional miRNAs that might be linked to miR172 - miR159 (Achard et al., 2004; Millar and Gubler, 2005; Allen et al., 2007b; Palatnik et al., 2007; Reyes and Chua, 2007; Alonso-Peral et al., 2010) and miR167 (Wu et al., 2006; Ru et al., 2006). The targets of miR159 have been discovered to be the GAMYB-like genes *MYB33* and *MYB65* (Alonso-Peral et al., 2012; Achard et al., 2004; Millar and Gubler, 2005; Allen et al., 2007b; Palatnik et al., 2007; Reyes and Chua, 2007; Alonso-Peral et al., 2010) while *ARF6* and *ARF8* are targeted by miR167 (Nagpal et al., 2005; Wu et al., 2006; Ru et al., 2006). Interestingly, these two post-transcriptional regulatory nodes form part of the signaling cascade of perhaps the two most important hormones controlling growth and development, auxin and gibberellin (GA). The miR167-ARF node is suspected of modulating the expression of auxin responsive genes, and the miR159-MYB node seems to transduce gibberellin (GA) signaling. Unfortunately, we have only recently isolated T1 transgenic lines for *OP::MIM167* and are currently crossing these lines to the proper *LhGAL4* driver lines. Therefore, we are currently unable to draw any conclusions about

miR167 and its role in valve development. We have, however, isolated T1 transgenic lines for *OP::MIM159* and have crossed them to the proper *LhGAL4* driver lines (details below).

The lack of miR159-regulation, by either mutating the miR159 encoding genes or misexpressing miR159-resistant versions of *MYB33/MYB65,* leads to pleiotropic developmental defects (Alonso-Peral et al., 2010; 2012; Rubio-Somoza and Weigel, 2011), which suggests the importance of this regulatory node during plant development. This is not surprising since the miR159-MYB node has been implicated in mediating different GA developmental responses such as meristem development, growth and cell proliferation, male sterility, and/or programmed cell death (Millar and Waterhouse, 2005; Reyes and Chua, 2007; Alonso-Peral et al., 2010; 2012).

Thus, we decided to take a look at the siliques of *miR159a,b* double mutants. We observed that after fertilization *miR159a,b* fruits are smaller when compared to those of wild-type plants (Figure 11B), however replum and valve margin seemed normal (data not shown). However, as mentioned previously, miR159-MYB regulates male fertility so we wondered whether a reduction in the total amount of fertility might be the cause of the resulting fruit valve phenotype. To rule out this possibility we pollinated emasculated *miR159a,b* pistils with wild-type pollen and observed a reduction in the final size of the fruits (data not shown). We also went one step further and specifically lowered the amount of endogenous miR159 in valve tissue using the *OP::MIM159* construct we generated and crossed it to the valve-specific driver line *FUL::LhGAL4*. Although the flowers from the resulting F1 crosses looked normal, fruit were smaller and resembled those of *arf6* -/- *arf8* +/- sesquimutant plants and *ful-6*. These results suggest that miR159 likely contributes to normal valve development and growth by repressing *MYB33* and *MYB65* in valve tissue, which might have a deleterious effect on valve growth.

Using Yeast-1-Hybrid to identify putative upstream regulators

Utilizing the yeast 1-hybrid protocol as shown in Figure 12, we used multiple transcription factor libraries to identify putative upstream regulators of *MIRNA*s. Each *MIRNA* promoter was inserted into the pLacZ vector and transformed into yeast. Using a machine developed in Jose Pruneda's lab, each promoter was screened using a high-throughput approach, where many transcription factors bound to activators were mixed with yeast containing the promoter of interest, incubated, and fold of induction calculated.

For the *MIR172C* promoter, 163 raw putative transcription factors were identified. Since the *MIR390* promoter is so large (~2500bp), we split the promoter region into 5 overlapping fragments before transforming into yeast. A total of 650 raw candidates were identified for the five fragments, of which 131 were from the 1st fragment, 127 from the 2nd fragment, 168 from the 3rd fragment, 162 from the 4th fragment, and 62 from the 5th fragment.

Filtering and modeling putative regulators of *MIR172C* and *MIR390* in Cytoscape

After obtaining the putative transcription factors for *MIR172C* and *MIR390*, we applied a series of filters in order to determine the best-fit transcription factors that may be involved *MIR172C* or *MIR390* regulation. The first filter used was to discard all transcription factors whose fold of induction was less than 4.0 when compared to the control (the control had a fold of induction = 1.0). This left 62 putative regulators for the *MIR172C* promoter while for the *MIR390* promoter, 109 putative regulators were left.

To determine if a transcription factor had negative regulation on the *miRNA* promoter, we screened for the presence of ethylene-activated-response (EAR) motifs. Transcription factors containing EAR motifs have been discovered to negatively regulate their target genes (Kagale et al., 2010). Of the 62 putative regulators of *MIR172C*, 9 contained EAR motifs (Figure 14). Of the 109 putative regulators of *MIR390*, 6 contained EAR motifs (Figure 18).

Another filter applied was identifying where the putative transcription factors were being expressed. Using the Arabidopsis eFP browser (Winter et al., 2007), each transcription factor was screened and those without expression patterns in fruit, specifically valve tissue, were discarded. For the *MIR172C* screen, 24 putative regulators remained, of which 2 regulators contained EAR motifs. For the *MIR390* screen, 52 putative regulators remained, of which 6 contained EAR motifs. After the filters were applied, all putative transcription factors were imported into the network analysis program, Cytoscape. In Cytoscape, regulatory networks for *MIR172C* and *MIR390* were generated with transcription factors organized using the force-directed weighted function, using fold of induction as the sorting factor (Figures 14, 15, 18, 19).

Modeling developmental expression of *MIR172C* and *MIR390A* putative regulators

Genevestigator software was used to analyze the expression of the putative regulators throughout the different stages of development. Specifically, we were interested in the expression of the identified putative transcription factors immediately before and during fruit development. After the initial filtering described above, all putative transcription factors were run through Genevestigator (Figures 16, 17, 20-22). These expression patterns were then compared to expression patterns of miRNA-targeted transcriptions factors involved in fruit development, such as *ARF6, ARF8, AP2, FUL, MYB33*, and *MYB65* (Figure 13). Expression patterns of many identified putative regulators mirrored those of known transcription factors involved in fruit development, suggesting that they play a role in fruit development.

Reconstructing the regulatory network

The bioinformatics work described above was then used to generate a working network of our current understanding of the developmental pathway of fruit in *Arabidopsis* (Figure 23). Interestingly, there were four common putative transcription factors shared between *MIR172C*

and *MIR1390*. Experiments are currently being performed to identify the involvement of these four transcription factors. A few key transcription factors we found to be of interest for *MIR172C* were *STYLISH 2 (STY2), CAULIFLOWER (CAL1)* and *TSO1*. For the *MIR390* promoter, we found the transcription factor *YABBY3 (YAB3)*. Another member in the Yanofsky lab has been working with miR390 and has found that the *YAB3* transcription factor is involved in fruit development (Bailey, unpublished). By combining the high-throughput method of identifying putative transcription factors, applying multiple filter attributes, and using the expression profile of each transcription profile during development has allowed us quickly identify multiple transcription factors in which we can design experiments and use classical approaches to verify their involvement in fruit development.

DISCUSSION

As previously discussed, we know that AP2 acts to prevent valve margin and replum overgrowth by negatively regulating the expression of valve margin and replum identity genes (Ripoll et al., 2011). Curiously, however, even though *AP2* messenger is expressed in valve tissue, *ap2* fruit valves do not seem to be affected, suggesting that somehow, AP2 protein is not made in valve tissue. Previous research performed by other members of our laboratory and the results presented in this study allowed us to conclude that, miR172 prevents AP2 function in valve tissue, much like how it happens during flowering time regulation and floral development. Interestingly one of the miR172 encoding genes, *MIR172C*, shows specific expression in carpel and valve tissue. At this point we wanted to elucidate the transcriptional regulation of MIR172 genes during fruit development. Our lab previously found that *FUL*, which is pivotal in valve development, directly regulates *MIR172C* expression in valve tissues (Ripoll et al., unpublished) by recognizing CArG-Box motifs within the *MIR172C* promoter. By following classical and high throughput techniques, we were able to identify additional upstream regulators of *MIR172* genes.

AuxREs within the *MIR172C* promoter are involved in positively regulating *MIR172C* expression

Beside the CArG-box motifs, our *in silico* analyses revealed the presence of two AuxREs regulatory motifs located 696bp and 707bp upstream of the transcriptional initiation site of the *MIR172C* gene. Our TAT experiments and expression assays in Arabidopsis transgenic lines showed that the lack of both AuxREs in the *MIR172C* promoter almost abolished the reporter signal in *MIR172C*$^{AuxRE-/-}$*::GUS* (Figure 9 and Figure 10). These results indicate the importance of the AuxRE regulatory motifs in activating *MIR172C* expression.

ARF6 and ARF8 Likely Regulate *MIR172C*

ARF6 and ARF8 have been found to play a role in floral organ development and flower maturation. In *arf6 arf8* double mutants, fruit are small and fail to fully elongate after fertilization (Nagpal et al., 2005; Wu et al., 2006; Tabata et al., 2010). This defect resembles that of *FUL>>AP2*^{miR172R} or *35S::MIM172* (Figures 7B and 7D). These phenotypic similarities and the significant reduction of *MIR172C* expression in fruit valve tissue in *MIR172C*$^{\Delta auxRE-/-}$*::GUS* transgenic plants (Figure 10C) made us wonder whether *ARF6* and *8* were regulating *MIR172C* expression in fruits. Our expression analysis confirmed this extreme as *MIR172C::GUS* activity was reduced in *arf6 -/- arf8 +/-* sesquimutant and virtually abolished in *arf6 arf8* double mutant fruit (Figure 9). All together, our data strongly suggests *MIR172C* expression is regulated by *ARF6* and *ARF8* in fruits. We are currently generating constructs to perform a ChIP-qPCR assay to determine if this regulation is direct ~~or not~~.

miR159 and miR167 Likely Participate in Valve Development

Two additional miRNAs we analyzed were miR159 and miR167. These two post-transcriptional regulatory nodes form part of the signaling cascade of perhaps the two most important hormones controlling growth and development in plants, auxin and gibberellin (GA) (Achard et al., 2004; Millar and Waterhouse, 2005; Wu et al., 2006; Allen et al., 2007a; Palatnik et al., 2007; Reyes and Chua, 2007; Alonso-Peral et al., 2010). The miR167-ARF node is suspected of modulating the expression of auxin responsive genes (Nagpal et al., 2005; Ru et al., 2006; Wu et al., 2006), and the miR159-MYB node seems to transduce signaling, which modulates meristem development, growth and cell proliferation, male sterility, and programmed cell death (Millar and Gubler, 2005; Reyes and Chua, 2007; Alonso-Peral et al., 2010; 2012). Studies on these two miRNAs and their roles in fruit development are currently being performed in our lab following different strategies, for example, target MIMICRY technology (Todesco et

al., 2010). Using MIM technology in combination with the two-component system we are able to specifically lower the amount of miR167 and/or miR159. For example, we can lower miR159 and miR167 expression in valve tissue using the *FUL::LhGAL4* transgenic driver (see Introduction for more details).

We are currently crossing our recently isolated T1 *OP::MIM167* lines to *LhGAL4* driver lines to analyze any phenotypic variations from wild-type plants. Based on our data of AuxRE domain roles in *MIR172C* expression, the ARF6 and ARF8 transcription factors are involved in the regulation of *MIR172C*, and knowing that *ARF6* and *ARF8* are targets of *MIR167*, we suspect by lowering the amount of miR167 activity, expression of *ARF6* and *ARF8* will be increased, resulting in an increase in *MIR172C* expression and increased valve sizes.

The lack of miR159-regulation, by either mutating the miR159 encoding genes or misexpressing miR159-resistant versions of *MYB33/MYB65,* leads to pleiotropic developmental defects (Alonso-Peral et al., 2010; Rubio-Somoza and Weigel, 2011; Alonso-Peral et al., 2012), which suggests the importance of this regulatory node during plant development. Looking at the siliques of *miR159a,b* double mutants, we observed that after fertilization *miR159a,b* fruits are smaller when compared to those of wild-type plants (Figure 11B), however replum and valve margin seemed normal (data not shown). As mentioned previously, miR159-MYB regulates male fertility (Millar and Gubler, 2005; Reyes and Chua, 2007; Alonso-Peral et al., 2010; 2012). Our data indicates that the reduced male fertility of *miR159a,b* is not involved in the reduced valve phenotype observed, as pollinating *miR159a,b* pistils with wild-type pollen did not rescue the valve defects (data not shown). Additionally, we specifically lowered miR159 in valves by crossing our *OP::MIM159* line to the valve specific driver line *FUL::LhGAL4*. The fruits in the resulting F1 plants (FUL>>MIM159) were very similar to those of *miR159a,b* plants. Interestingly, fruit FUL>>MIM159 phenotype resembled that of *arf6 -/- arf8 +/-* and *ful-6* mutants (Figure 7E and Figure 10C), which might suggest that the miR159-MYB node interacts

with *ARF6,8* and/or *FUL* to some degree. These results suggest that miR159 likely contributes to normal valve development and growth by repressing its targets, *MYB33* and *MYB65*, which may have a deleterious effect on valve growth.

A Bioinformatics Approach at the Gene Regulatory Network Surrounding Fruit Development

We proceeded to utilize bioinformatics tools to more efficiently identify putative transcriptional regulators of *MIRNA* genes. After identifying putative regulators, we could then use classical approaches to determine if the identified candidates were true regulators involved in fruit development.

Utilizing yeast one-hybrid assays, we were able to identify hundreds of putative regulators of *MIR172C* and *MIR390*. While it is impossible to determine if the identified transcription factors are positive or negative regulators until experiments are performed, we conducted a screen for EAR motifs within the protein sequence of the identified transcription factors. Many studies have shown that transcription factors containing EAR motifs are negative regulators of their targets (Kagale et al., 2010; Kagale and Rozwadowski, 2010). Utilizing this knowledge, we are able to determine if an identified transcription factor is a likely repressor.

Due to the large amount of putative regulators identified (163 for *MIR172C* and 650 for *MIR390A*), a set of filters was necessary to limit the number of putative regulators to be analyzed. The first filter was to discard putative regulators with folds of induction less than 4.0, which might decrease the likelihood it is a direct regulator of the promoter in question.

The *Arabidopsis* eFP browser was used as the second screen to determine where a transcription factor was expressed in the plant (Winter et al., 2007). Since our study was focused on fruit development, we used the eFP browser to screen for putative transcription factors with expression in fruit tissues, specifically in valves. Transcription factors that were not expressed in

fruits were discarded. The limiting factor of using the eFP browser was that there was no information detailing during which stage of development expression was noticed. This drawback was solved through the use of the Genevestigator tool (detailed below). The filtered data was then passed into the Cytoscape program to generate an easy to read network model.

Genevestigator was used to analyze the expression levels of identified putative regulators during different stages of plant development (Hruz et al., 2008). In order to establish a baseline, an expression profile of known transcription factors involved in fruit was generated (Figure 13). For all of the transcription factors, expression increased starting from inflorescence development and remained elevated until fruit maturation. Identified putative regulators were then run through Genevestigator and their expression profiles compared to the baseline. If the identified putative regulators exhibited similar expression patterns, they are considered likely to be involved in fruit development and experiments can be designed to verify their role in fruit development.

TSO1 **and** ***CAL*** **are likely candidates for** ***MIR172C*** **regulation**

While many transcription factors were identified after the filtering process, two transcription factors identified from the *MIR172C* data stood out: *TSO1* (Chinese for 'ugly'*)*, and *CAULIFLOWER (CAL)*, with folds of induction of 15.3 and 4.2, respectively. *TSO1* has been found to play a role in the formation of petals, stamens, and carpels (Liu et al., 1997; Hauser et al., 1998) and *CAL* has been found to play a redundant role with *FUL* in regulating meristem growth (Ferrandiz et al., 2000b).

TSO1 is a member of the CPP transcription factor family, which is widely present in plants and animals and is known to play an important role in reproductive tissue development and cell division (Yang et al., 2008). *TSO1* stands out from our identified candidates of *MIR172C* regulators because of the high fold of induction, high level of expression during fruit development, expression specifically in fruit valves, and the fruit phenotype of *tso1* mutants.

Because our research revolves around fruit development, and *TSO1* has been found to play a role in carpel formation, we are interested in elucidating the role of *TSO1* in reproductive development concerning *MIR172C* regulation. To do this, we can cross our *MIR172::GUS* transgenics to *tso1* mutants and analyze the expression pattern.

CAL is a member of the MADS-box transcription factor family and along with *FUL*, has been shown to regulate meristem identity architecture (Ferrandiz et al., 2000b). MADS-box transcription factors have been shown to form homodimers or heterodimers when binding to DNA (Huang et al., 1996), suggesting that perhaps *CAL* and *FUL* form a heterodimer and bind to the same CArG-box cis-regulatory motif on the *MIR172C* promoter. To test if this is true, one of the experiments we can perform is to generate a *CAL::GFP* construct to perform a ChIP assay, much like the one that was performed for *FUL*. If similar fold of enrichments are found for *CAL* compared to the *FUL* ChIP assay, this would indicate that *FUL* and *CAL* do form a heterodimer and synergistically regulate *MIR172C*.

Future steps

As research in fruit development advances, we are made aware that the story of the regulatory network behind fruit development is largely unfinished. Through the use of high-throughput techniques such as deep sequencing and expression profile analysis, we can quickly elucidate potential factors involved in fruit development. We can then use the data obtained and combine it with classical biological approaches to conclusively determine the roles of identified genes in the regulatory network. Individually, each method of approach has significant drawbacks. Using classical approaches often takes a long time while high-throughput approaches only generate hypothetical data sets that need to be verified experimentally. Combining the two approaches offsets the drawbacks of both methods and our lab has used this combined method to successfully identify multiple transcription factors that potentially regulate miRNAs involved in

fruit development. The results we obtained provide further insight into the complexity of gene regulation in something as “simple” as fruit development and open the door to additional research opportunities.

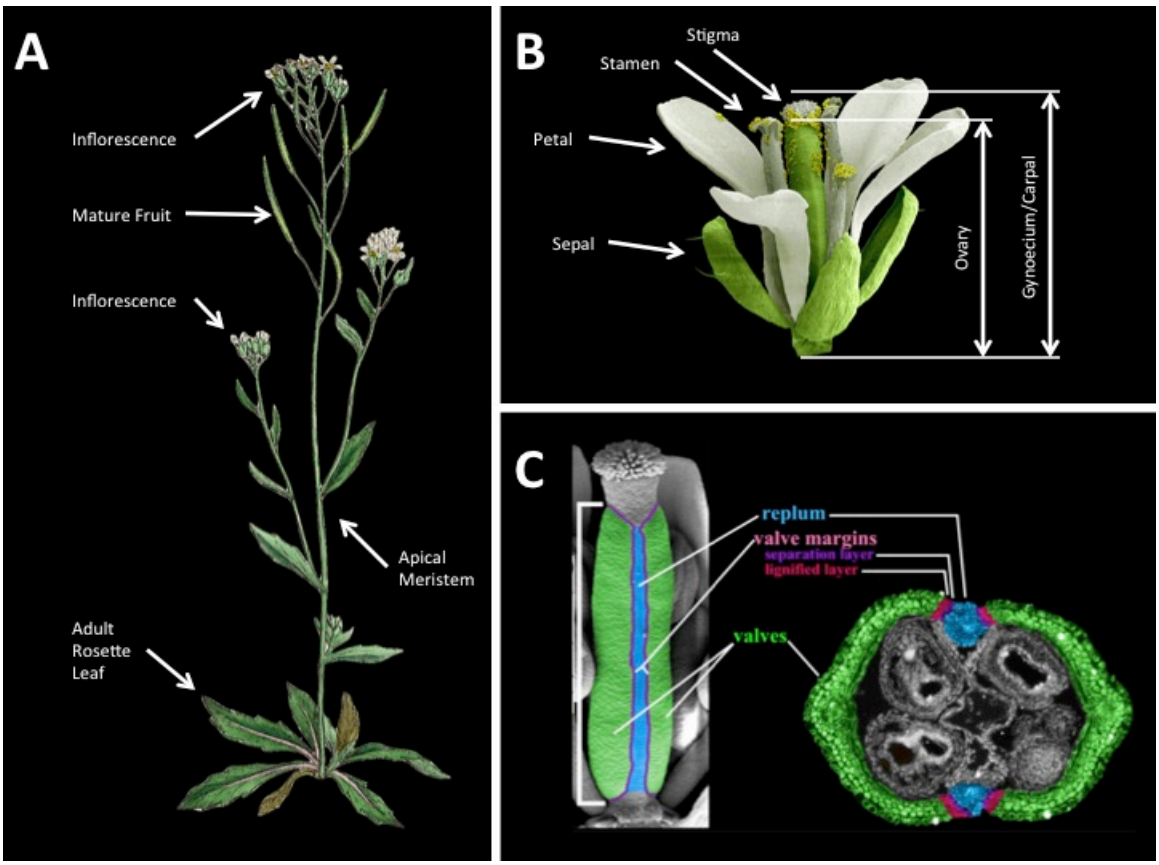


Figure 1. Basic anatomy of *Arabidopsis thaliana*
(A) Basic anatomy of whole plant showing central shoot, lateral shoots, and inflorescences.
(B) Flower anatomy depicting the four whorls of organs.
(C) On the left, a stage 12 gynoecium in which the territories that conform the mediolateral axis of the ovary have been color-coded. Valves are highlighted in green, replum in blue, and valve margins in purple. On the right a cross section of a mature fruit illustrating the outer tissues of the ovary.

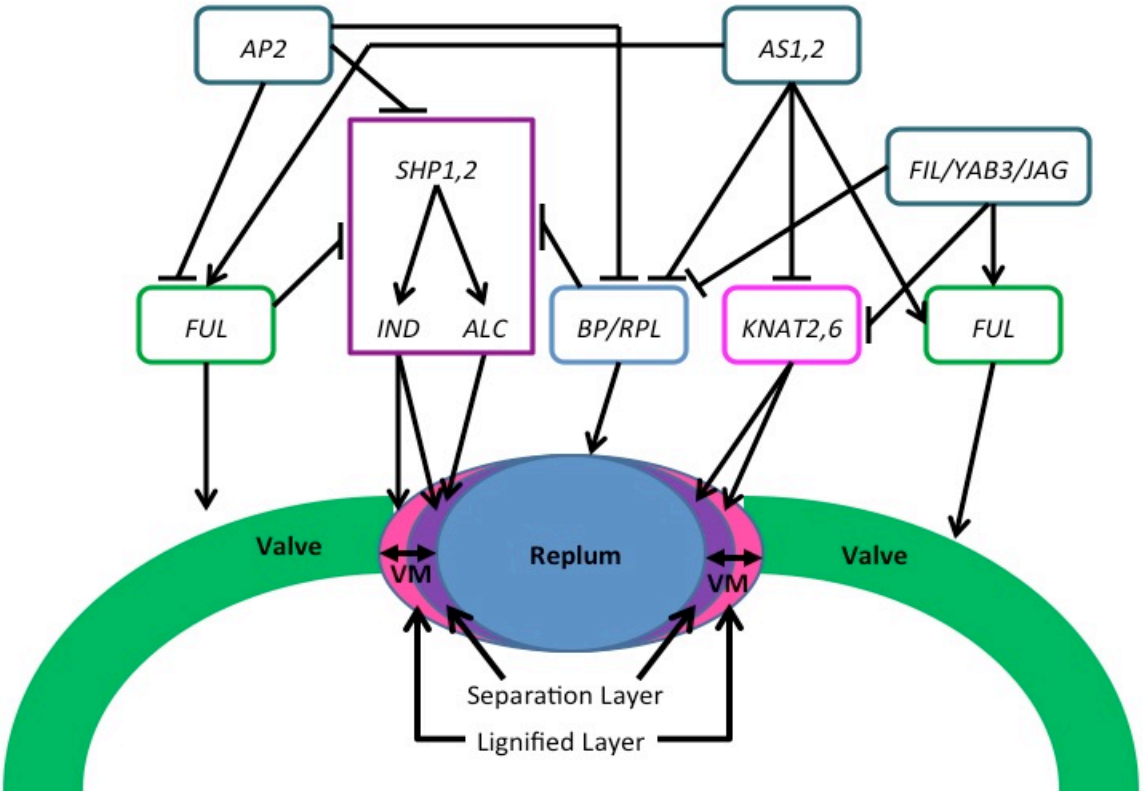


Figure 2. Current regulatory network controlling fruit development
The model shows participating regulatory genes and the genetic interactions that take place during fruit morphogenesis in *Arabidopsis*.

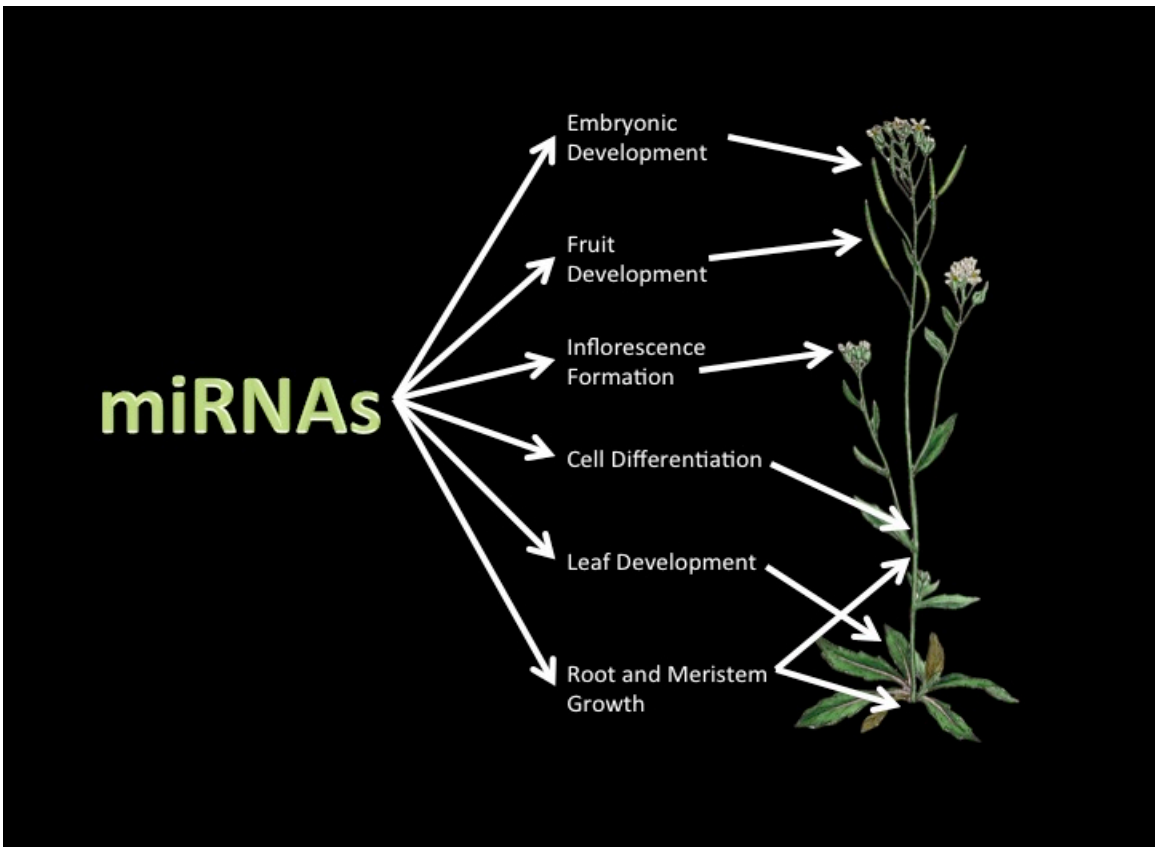


Figure 3. Importance of small RNAs in plant development
In plants, miRNAs and also ta-siRNAs, participate in different developmental processes during both the vegetative and the reproductive phases of growth.

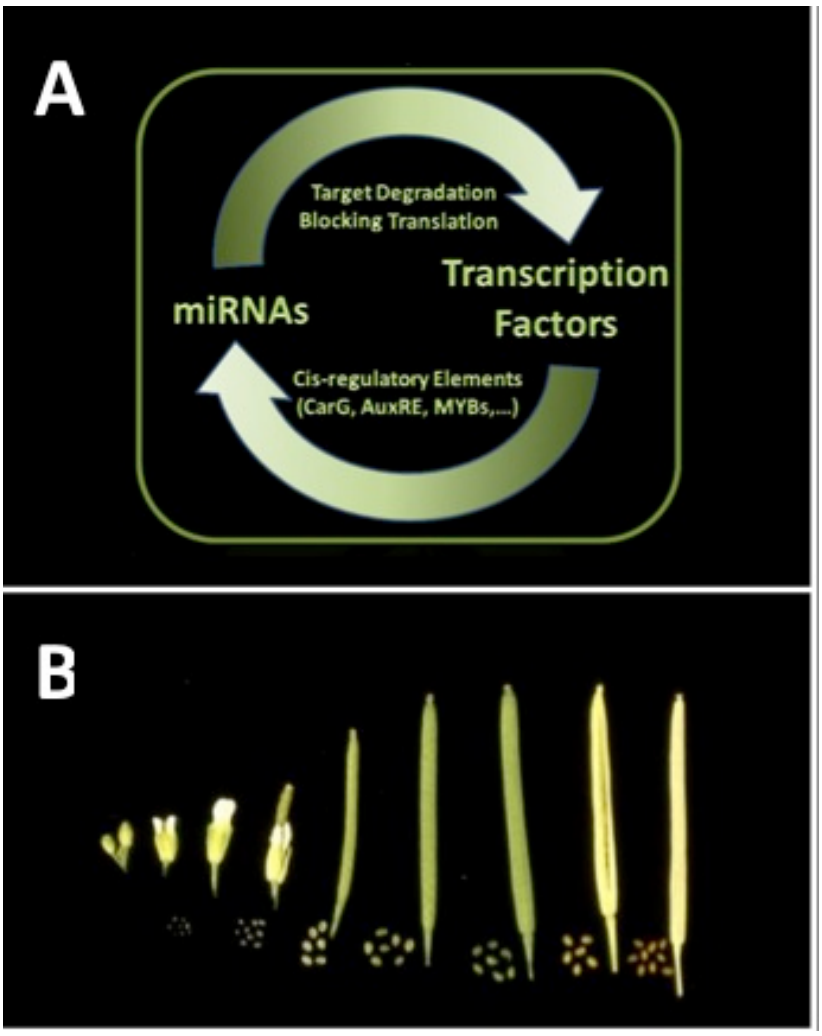


Figure 4. Fruit development as a platform to elucidate the connections between transcriptional regulation and post-transcriptional control mediated by miRNAs
(A) Model illustrating regulation of miRNAs and transcription factors. **(B)** Different stages of fruit development from inflorescence to fully developed fruit.

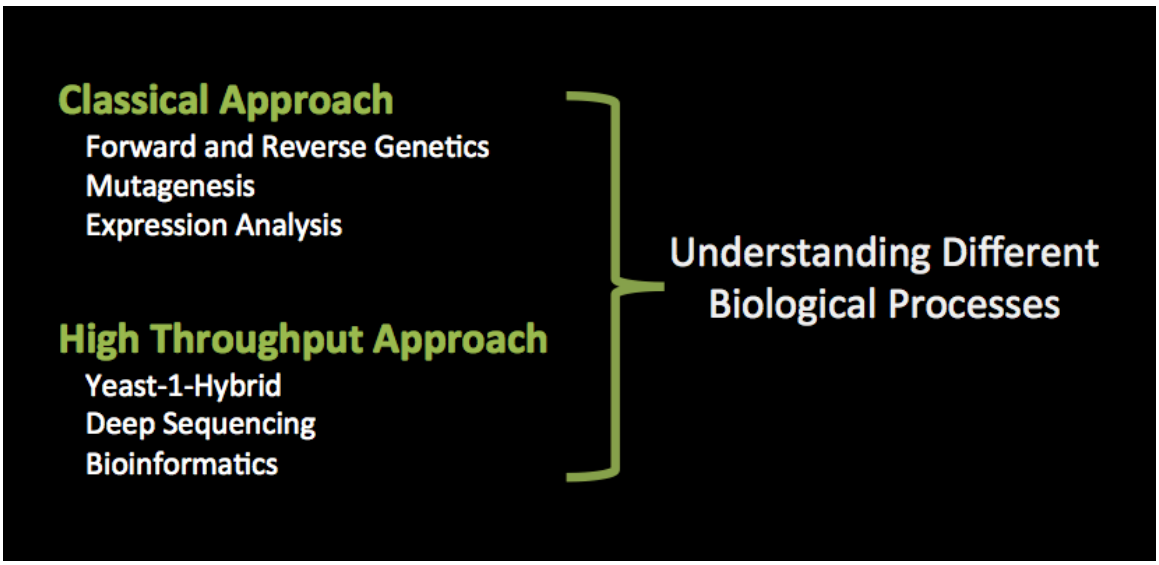


Figure 5. Combining classical and high throughput approaches to more efficiently dissect biological processes
Methodology used in this work to uncover novel transcriptional and post-transcriptional regulators of fruit development in *Arabidopsis*.

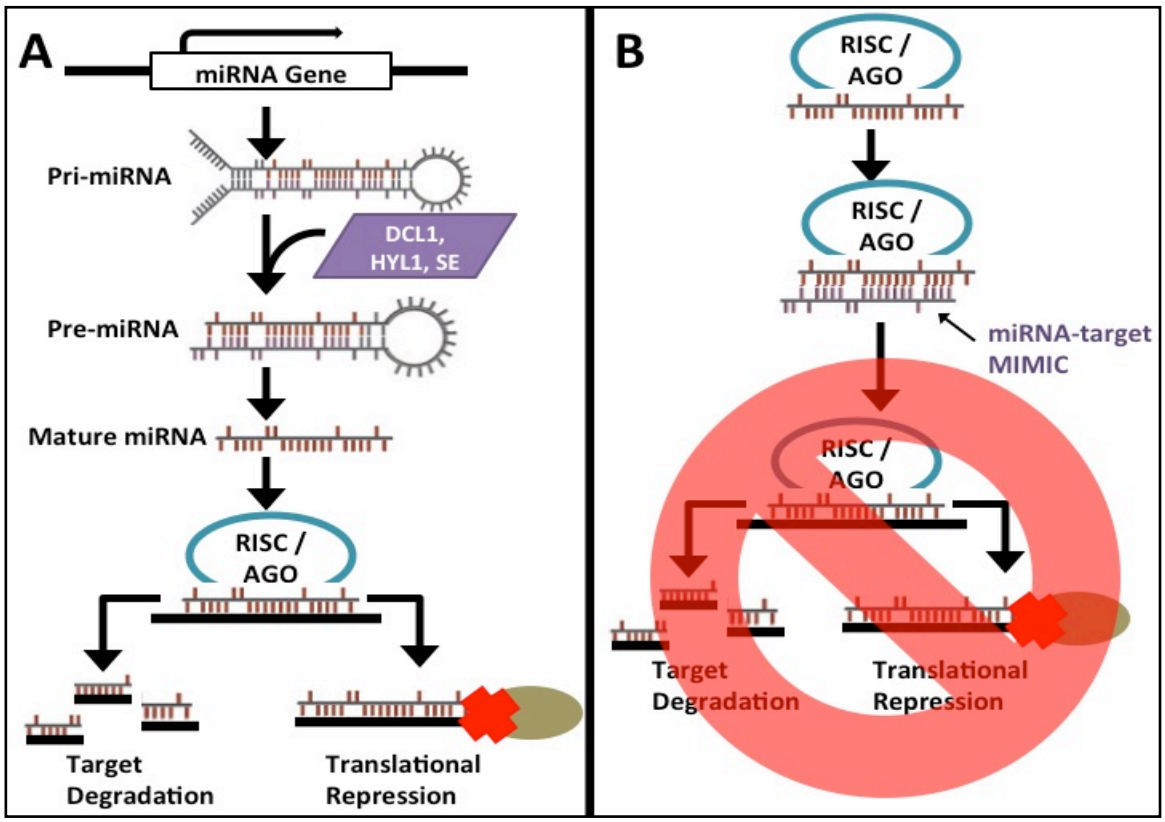


Figure 6. miRNA biosynthesis Pathway and miRNA target MIMICRY Technology
(A) Current model for small RNA biogenesis (miRNA and tasiRNAs). **(B)** Model for Inhibition of miRNA function by target mimicry nucleic acid molecules (*MIM*).

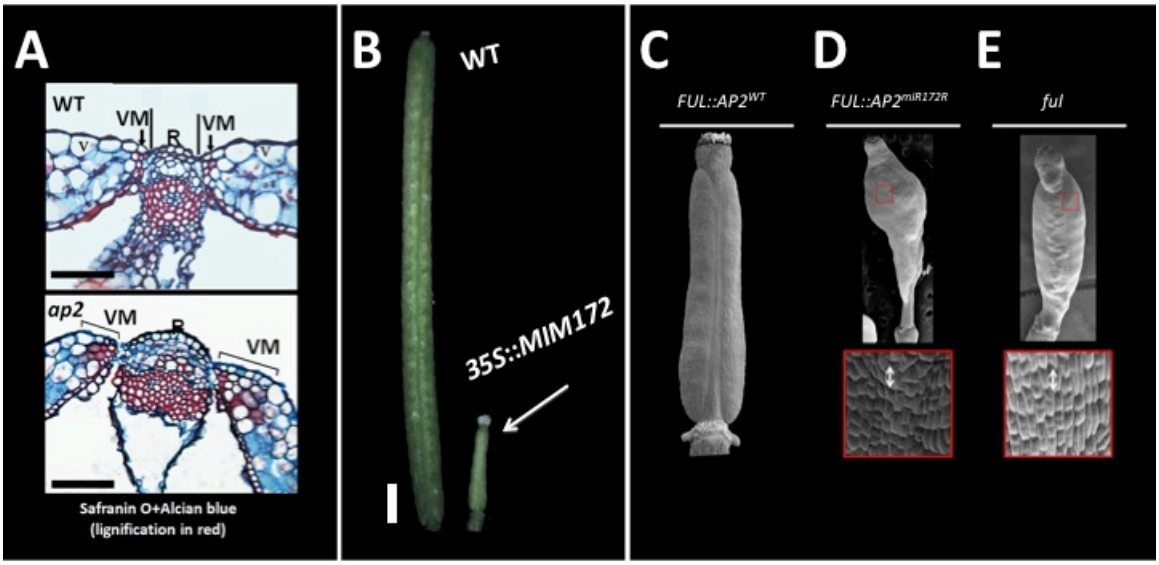


Figure 7. ***AP2*** **and miR172 participate in fruit development**
(A) In *ap2* fruits, replum and valve margin are enlarged indicating that *AP2* negatively regulates the growth of these two tissues (Ripoll et al., 2011) **(B)** Overexpression of *MIM172* results in fruit with decreased valve cell size. **(C-E)** Scanning electron microscope (SEM) images of *Arabidopsis* fruit. **(C)** Wild-type *AP2* cDNA expressed in valve tissue using *FUL* promoter. **(D)** miR172-resistant version of *AP2* cDNA expressed in valve tissue using the *FUL* promoter. **(E)** *ful* mutant fruit. Valve cell length between **(D)** and **(E)** is similar.

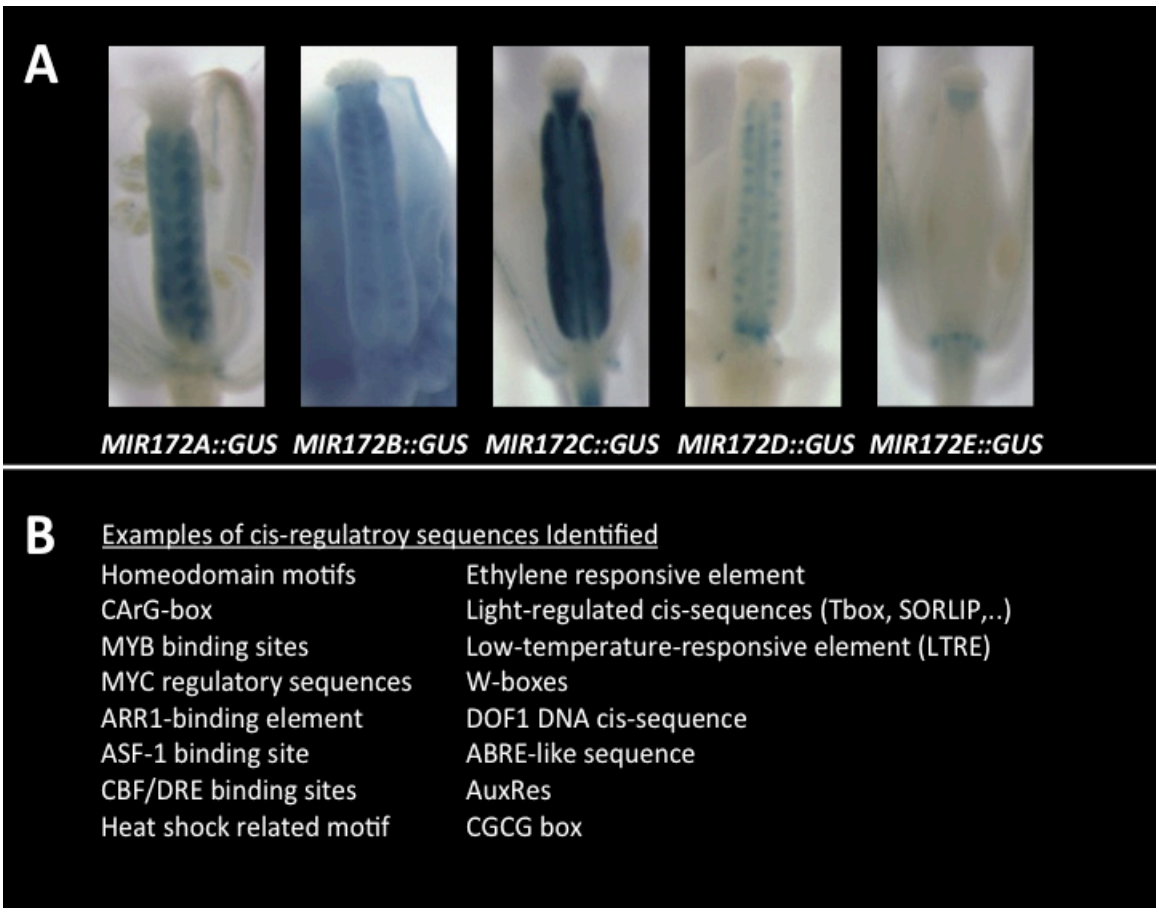


Figure 8. *GUS* Reporter lines generated for *MIR172* loci and cis-motifs identified within the regulatory regions
(A) Expression patterns for *MIR172*::*GUS* reporters (modified from Mai, 2008).
(B) Cis-regulatory motifs found within the regulatory region of *MIR172C*.

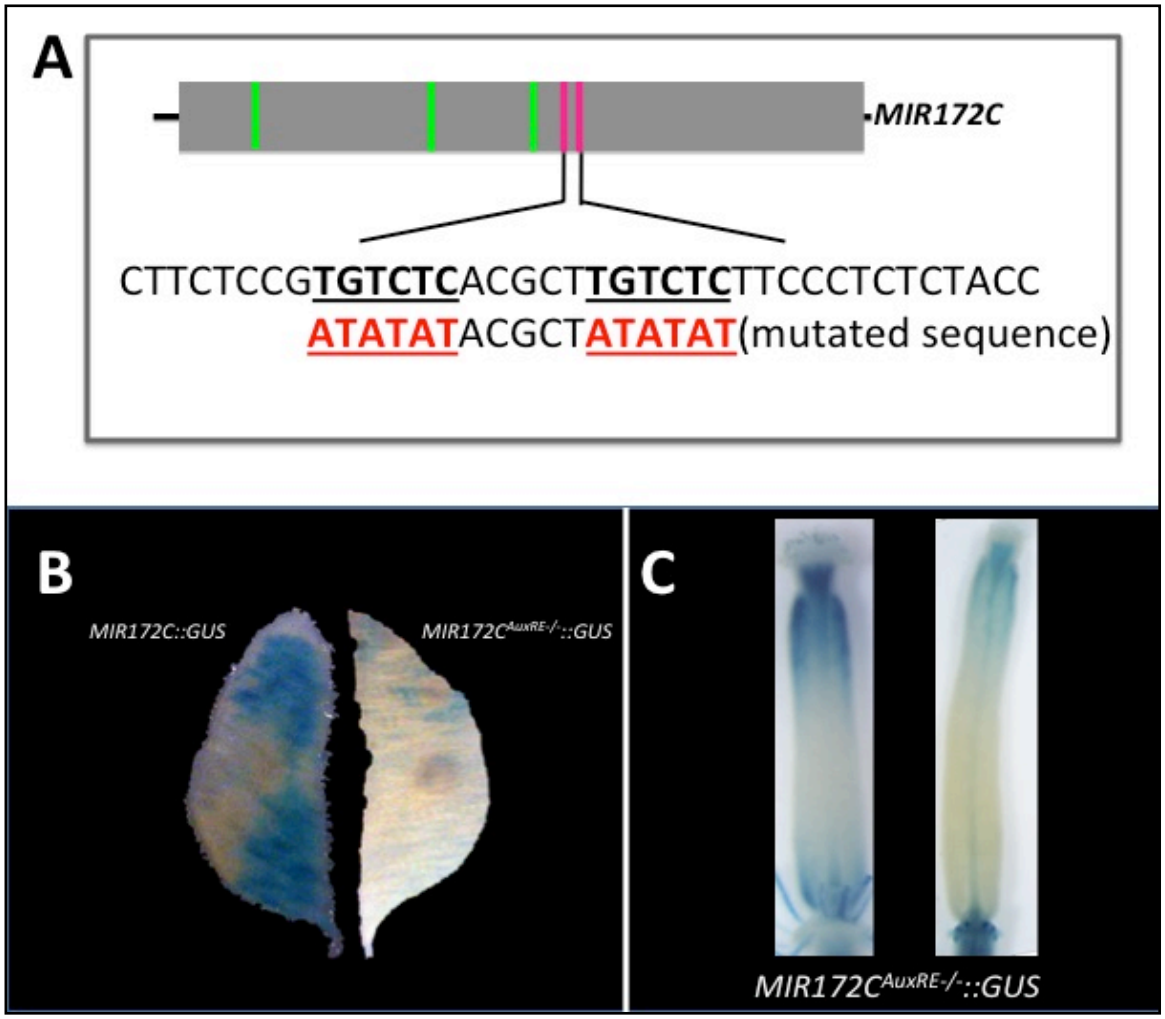


Figure 9. AuxRE motifs mediate positive regulation of *MIR172C* in valves
(A) *MIR172C* promoter highlighting in green the CArG-boxes and in pink the location of the AuxRE motifs. These motifs were mutated to determine their importance in regulating *MIR172C*. **(B)** Transient expression assay in tobacco leaves (TAT) using *MIR172C*^{WT}*::GUS* and *MIR172C*$^{AuxRE-/-}$*::GUS,* respectively. **(C)** Transgenic *Arabidopsis* plant harboring the *MIR172C*$^{AuxRE-/-}$*::GUS* construct.

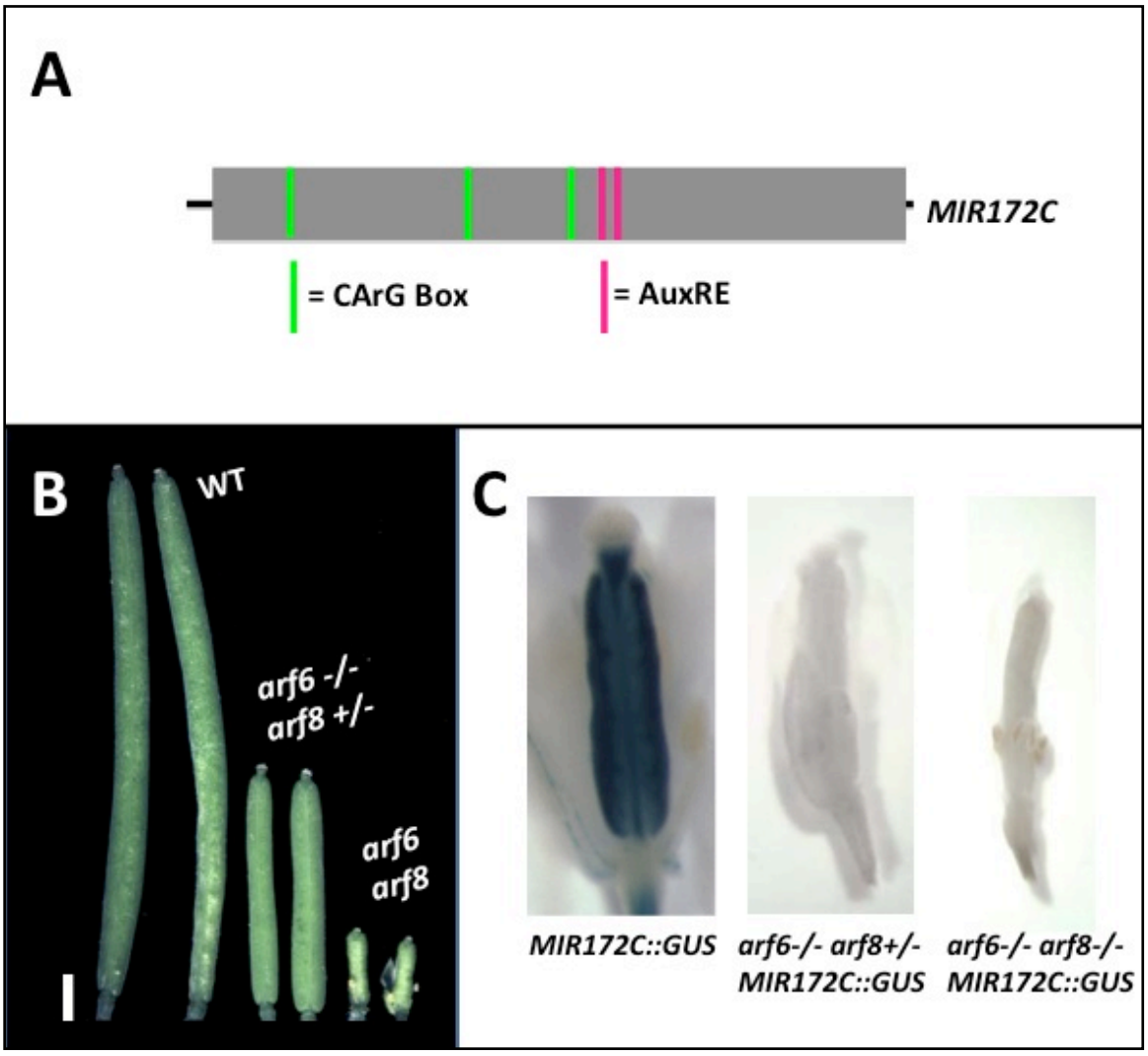


Figure 10. ***ARF6*** **and** ***ARF8*** **are the best candidates to positively regulate** ***MIR172C*** **through AuxRE motifs**

(A) *MIR172C* promoter analysis shows two Auxin Responding Elements (AuxREs, in purple) **(B)** Fruit phenotypes of wild-type, *arf6-/- arf8+/-* , and *arf6 arf8* double mutant fruits. **(C)** *MIR172C::GUS* expression pattern in wild-type , *arf6-/- arf8+/-*, and *arf6 arf8* fruits, respectively

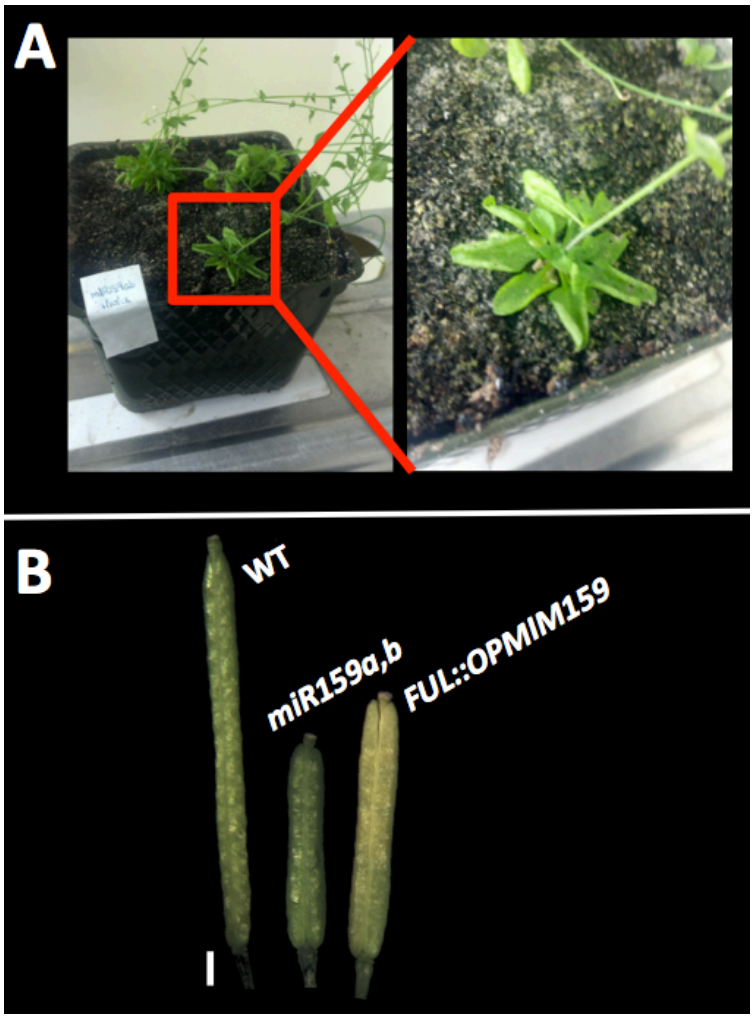


Figure 11. Effects of lack of miR159 function during fruit development
(A) Whole plant and close up of adult rosette leaves.
(B) Fruit of wild-type, *miR159a,b*, and *FUL>>OPMIM159* fruit.

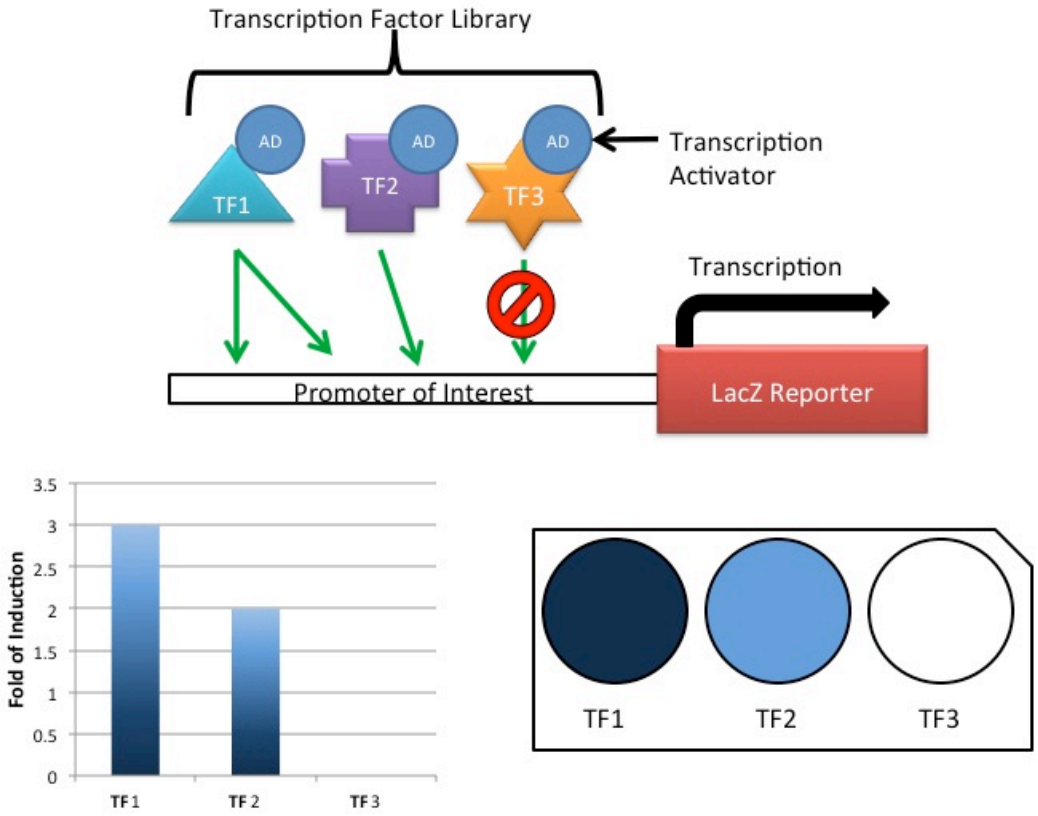


Figure 12. Promoter hiking methodology used in this work
Schematic representation of the Y1H approach used to identify upstream transcriptional regulators of miRNA encoding genes

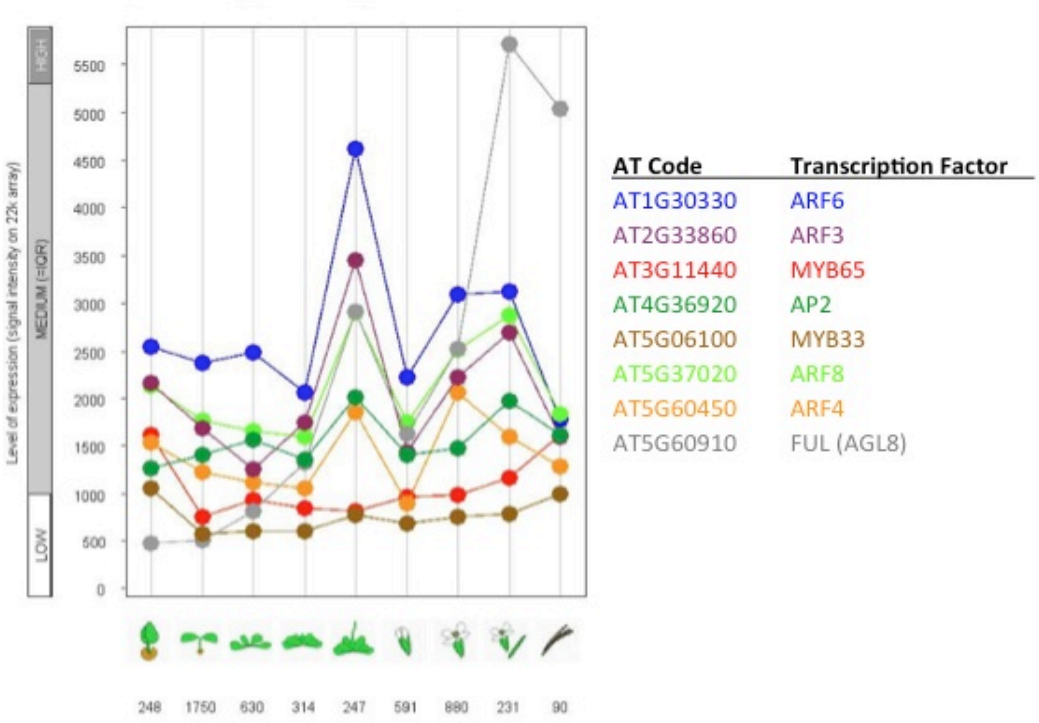


Figure 13. Expression profile of transcription factors controlling fruit development
Genevestigator expression profiles of known transcription factors regulating fruit development in *Arabidopsis*.

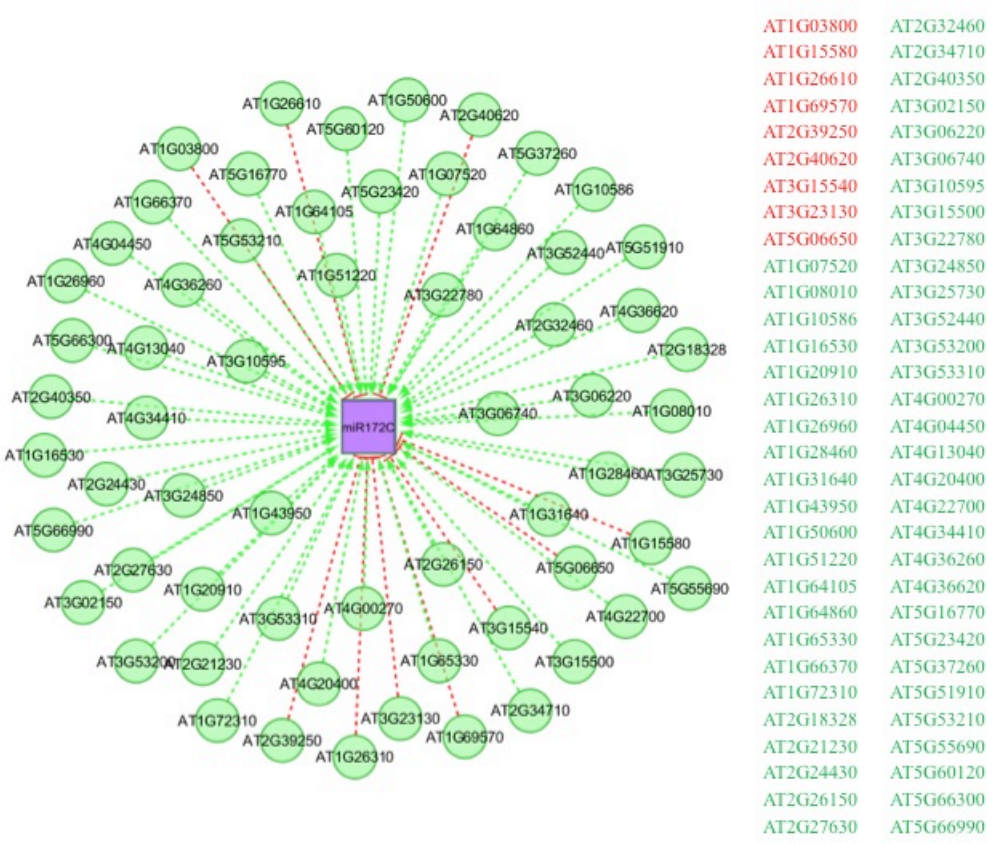
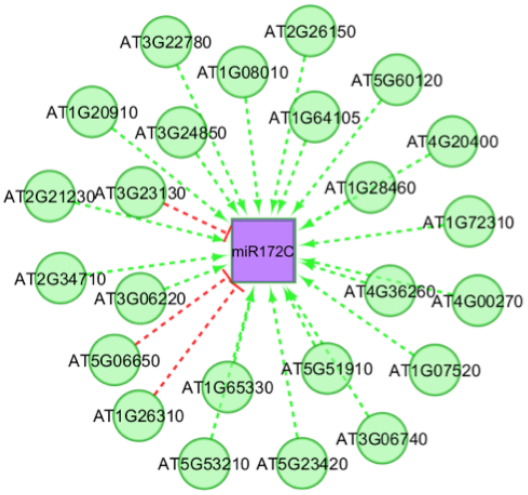
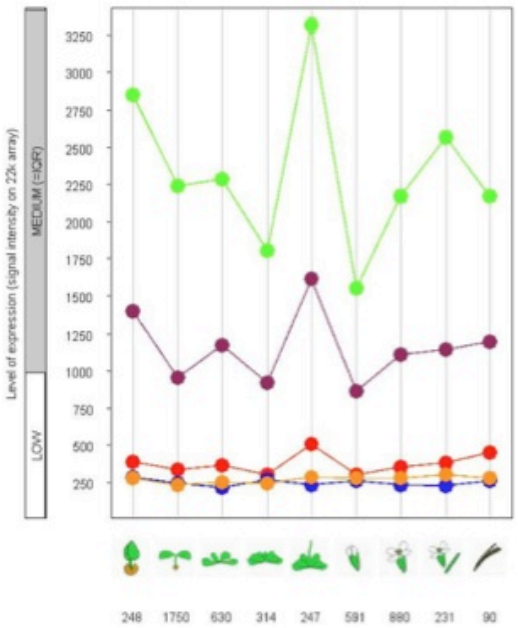


Figure 14. Putative upstream regulators of *MIR172C* obtained from promoter hiking
Y1H matches with folds of induction greater than 4.0. Genes in red indicate putative negative regulators. Genes in green indicate putative positive regulators.



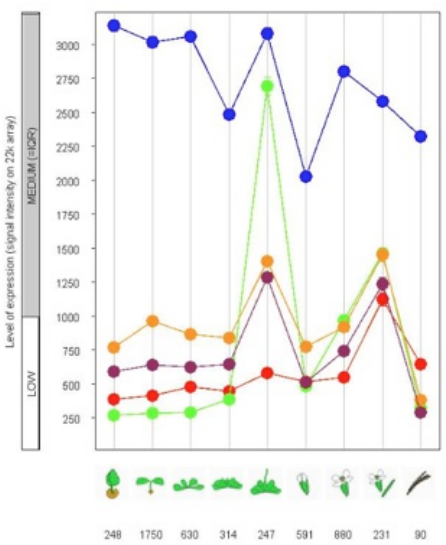
AT Code	TF Family	Name	Fold of Induction
AT4G36260		STY2/SRS2	25.4
AT3G22780	CPP	ATTSO1	15.3
AT5G51910	TCP		6.63
AT3G24850		DUF313	6.32
AT5G06650	C2H2	GIS2	6.17
AT3G06220	ABI3VP1		5.92
AT4G00270	GeBP		5.67
AT3G06740	C2C2-GATA	GATA15	5.61
AT5G23420		HMGB6	5.57
AT1G72310		ATL3	5.47
AT1G08010	GATA (C2C2)	GATA11	5.17
AT3G23130	C2H2	FLO10	4.7
AT4G20400	JUMONJI	JMJ14	4.5
AT5G53210	bHLH	SPCH	4.48
AT2G21230	bZIP		4.47
AT1G65330	MADS	AGL37	4.26
AT1G20910	ARID		4.24
AT1G26310	MADS	CAL	4.17
AT2G34710	HB	PHB-1D	4.17
AT1G07520	GRAS		4.17
AT2G26150		HSFA2	4.12
AT1G28460	MADS	AGL59	4.11
AT5G60120	AP2	TOE2	4.06
AT1G64105	NAC	ANAC27	4.05

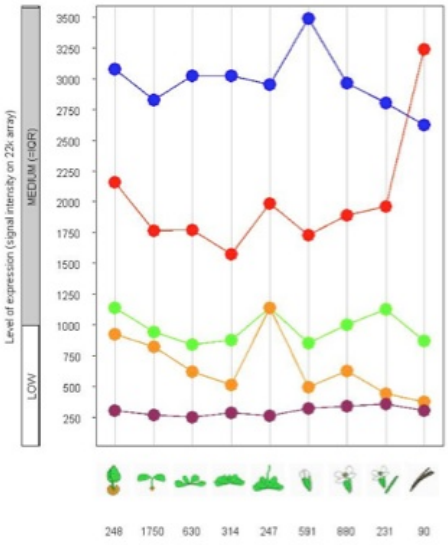
Figure 15. Filtered putative regulators of *MIR172C*
Y1H matches with folds of induction greater than 4.0 and displayed expression in fruit tissue in *Arabidopsis* eFP Browser. Genes in red indicate putative negative regulators. Genes in green indicate putative positive regulators.



AT Code	TF Family	Name	Fold of Induction
AT4G36260		STY2/SRS2	25.4
AT3G22780	CPP	TSO1	15.3
AT5G51910	TCP		6.63
AT3G24850		DUF313	6.32
AT5G06650	C2H2	GIS2	6.17

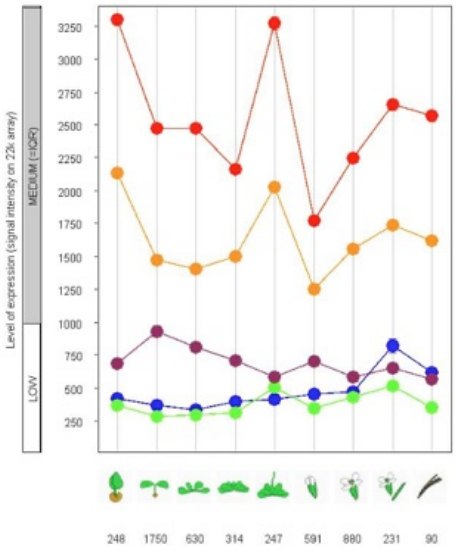
Figure 16. Genevestigator expression profile of top 5 candidates of *MIR172C*
Expression profiles of top 5 candidates of *MIR172C*. STY2 and TSO1 show expression profiles similar to known transcription factors that regulate *MIR172C*

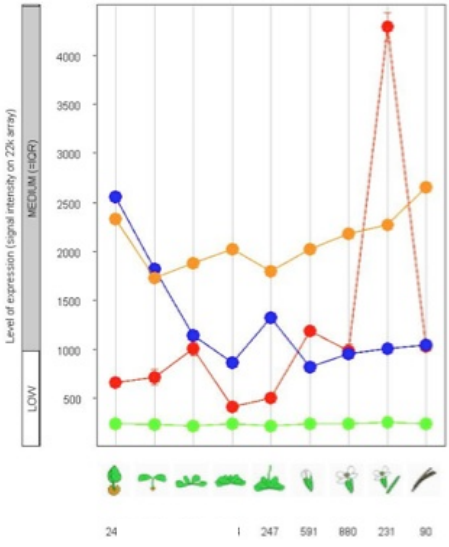




AT Code	TF Family	Name	Fold of Induction
AT3G06220	ABI3VP1		5.92
AT4G00270	GeBP		5.67
AT3G06740	C2C2-GATA	GATA15	5.61
AT5G23420		HMGB6	5.57
AT1G72310		ATL3	5.47

AT Code	TF Family	Name	Fold of Induction
AT1G08010	GATA (C2C2)	GATA11	5.17
AT3G23130	C2H2	FLO10	4.7
AT4G20400	JUMONJI	JMJ14	4.5
AT5G53210	bHLH	SPCH	4.48
AT2G21230	bZIP		4.47





AT Code	TF Family	Name	Fold of Induction
AT1G65330	MADS	AGL37	4.26
AT1G20910	ARID		4.24
AT1G26310	MADS	CAL	4.17
AT2G34710	HB	PHB-1D	4.17
AT1G07520			4.17

AT Code	TF Family	Name	Fold of Induction
AT2G26150		HSFA2	4.12
AT1G28460	MADS	AGL59	4.11
AT5G60120	AP2 (SINGLE DOMAIN)	TOE2	4.06
AT1G64105	NAC	ANAC27	4.05

Figure 17. Genevestigator expression profiles of putative regulators of *MIR172C*
Expression profiles of remaining identified putative regulators of *MIR172C*.

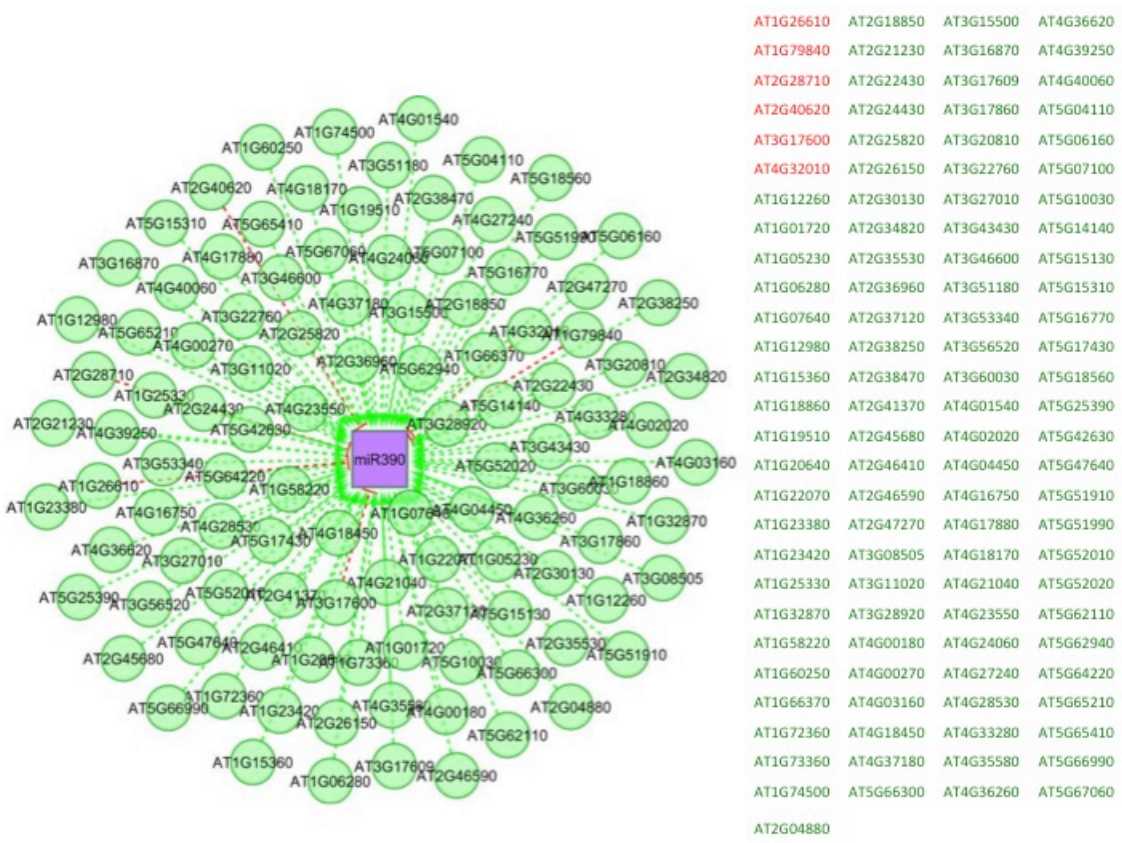


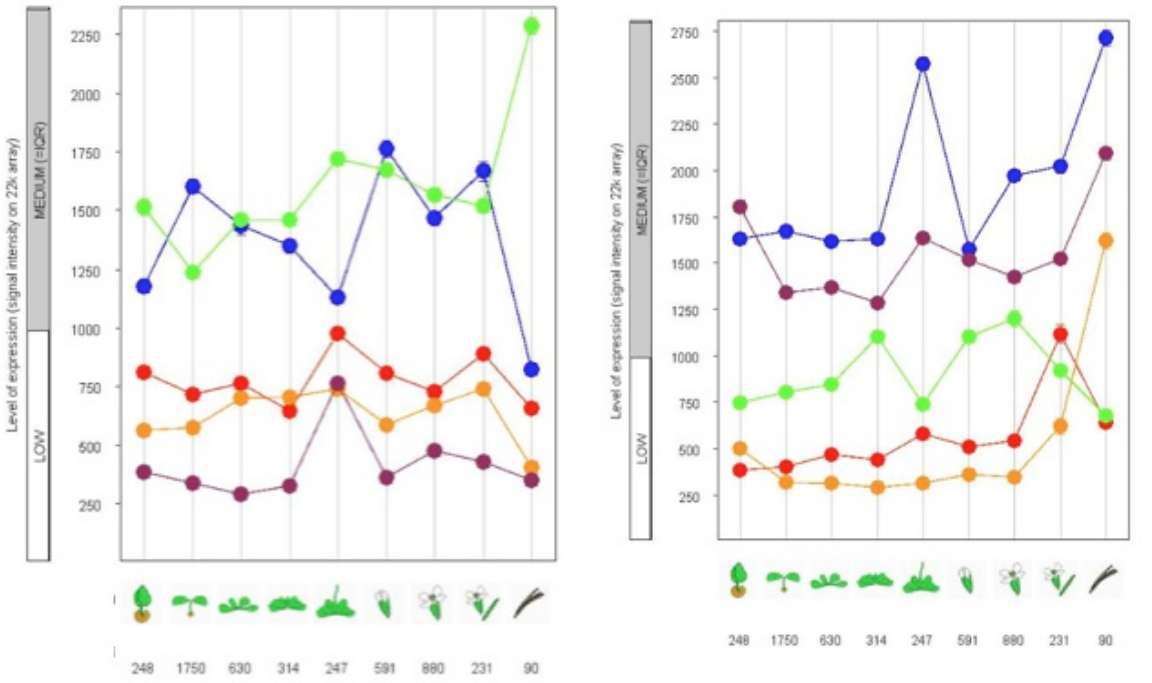
Figure 18. Putative upstream regulators of *MIR390* obtained from promoter hiking
Y1H matches with folds of induction greater than 4.0. Genes in red indicate putative negative regulators. Genes in green indicate putative positive regulators.

AT Code	TF Family	Name	Fold of Induction
AT5G62940	DOF (C2C2)	HCA2	14.5
AT2G45680	TCP		14.4
AT4G35580	NAC	NTL9	12.3
AT2G35530	BZIP	BZIP16	12.0
AT1G73360	HB	EDT1	10.6
AT4G00270	GeBP		9.2
AT1G58220	MYB-related		8.8
AT4G02020	SET	EZA1	7.7
AT1G05230	HB	HDG2	7.4
AT4G33280	ABI3VP1		7.3
AT3G17600	AUX-IAA	IAA31	6.3
AT3G16870	GATA (C2C2)	GATA17	6.2
AT5G51910	TCP		6.1
AT3G51180			5.9
AT5G47640	CCAAT	NF-YB2	5.9
AT5G51990	AP2-EREBP	CBF4 / DREB1D	5.9
AT4G17880	bHLH	MYC4	5.9
AT1G22070	BZIP	TGA3	5.7

AT Code	TF Family	Name	Fold of Induction
AT2G21230	bZIP		5.3
AT5G62110			5.2
AT2G37120	S1Fa-like		5.1
AT3G60030	SBP	SPL12	5.1
AT4G16750	ERF		5.1
AT3G53340	CCAAT	NF-YB10	5.0
AT4G24060	DOF (C2C2)		4.8
AT4G18450	AP2-EREBP		4.8
AT4G40060	HB	ATHB16	4.7
AT3G56520			4.6
AT2G38250	Trihelix		4.5
AT2G04880	WRKY	WRKY1	4.5
AT4G37180	G2-like		4.5
AT1G23420	YABBY (C2C2)	INO	4.5
AT5G64220	CAMTA		4.5
AT1G79840	HB	GL2	4.5
AT1G66370	MYB	MYB113	4.4

AT Code	TF Family	Name	Fold of Induction
AT5G25390	ERF	SHN3	4.4
AT2G26150		HSFA2	4.4
AT3G27010	TCP	TCP20 / PCF1	4.3
AT1G20640	RWP-RK		4.3
AT4G18170	WRKY	WRKY28	4.3
AT3G20810		JMJ30	4.3
AT4G00180	YABBY (C2C2)	YAB3	4.3
AT2G36960	MYB-related	Tkl1	4.2
AT1G23380	HB	KNAT6	4.2
AT5G52010	C2H2		4.1
AT5G06160	C2H2	ATO	4.1
AT5G18560	AP2-EREBP	PUCHI	4.1
AT2G18850			4.1
AT2G46590	DOF (C2C2)	DAG2	4.1
AT5G17430	AP2-EREBP	BBM	4.1
AT1G25330	bHLH	CES	4.1
AT2G22430	HB	ATHB6	4.0

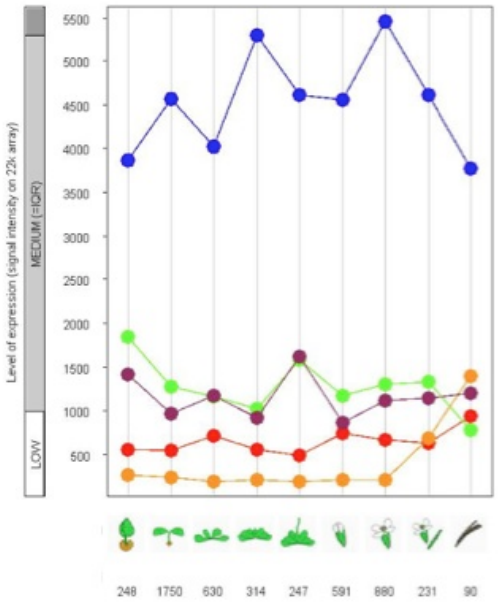
Figure 19. Filtered putative regulators of *MIR390*
Y1H matches with folds of induction greater than 4.0 and displayed expression in fruit tissue in *Arabidopsis* eFP Browser. Genes in red indicate putative negative regulators. Genes in green indicate putative positive regulators.



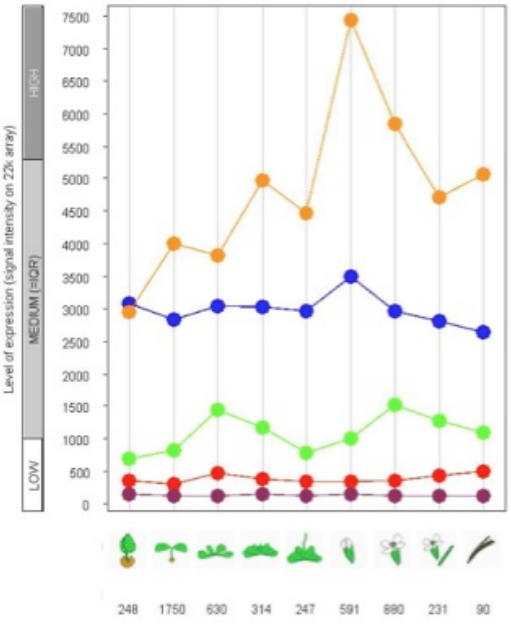
AT Code	TF Family	Name	Fold of Induction
AT5G62940	DOF (C2C2)	HCA2	14.5
AT2G45680			14.4
AT4G35580	NAC	NTL9	12.3
AT2G35530	BZIP	BZIP16	12.1
AT1G73360	HB	EDT1	10.6

AT Code	TF Family	Name	Fold of Induction
AT4G00270	GeBP		9.2
AT1G58220	MYB-related		8.8
AT4G02075	SET	EZA1	7.7
AT1G05230	HB	HDG2	7.4
AT4G33280	ABI3VP1		7.3

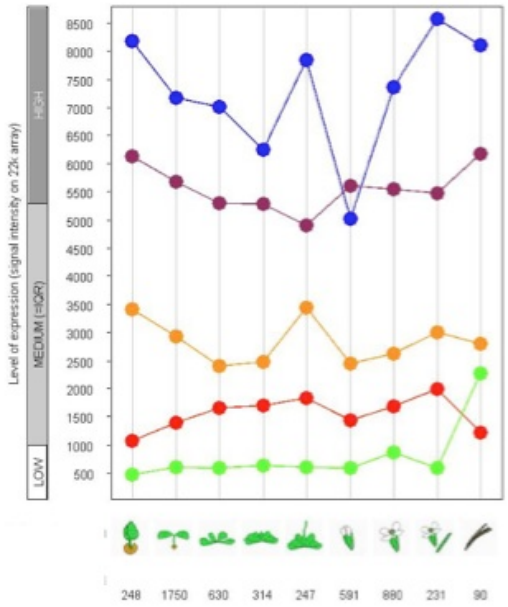
Figure 20. Genevestigator expression profiles of top 10 candidates of *MIR390*
Expression profiles of top 10 candidates of *MIR390*.



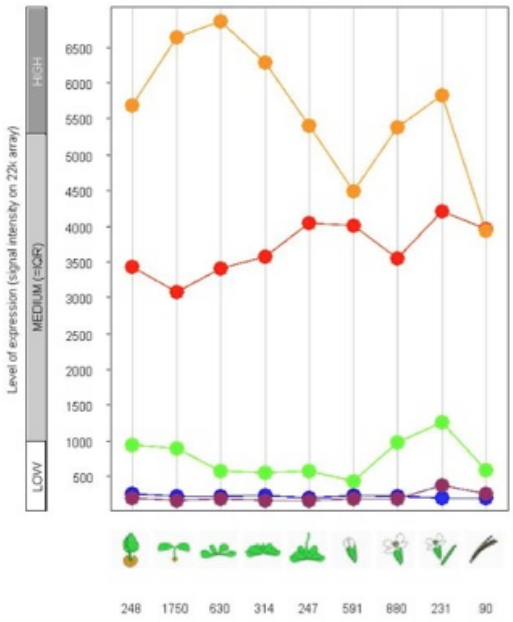
AT Code	TF Family	Name	Fold of Induction
AT3G17600	AUX-IAA	IAA31	6.3
AT3G16870	GATA (C2C2)	GATA17	6.2
AT5G51910			6.1
AT3G51180			5.9
AT5G47640		NF-YB2	5.9



AT Code	TF Family	Name	Fold of Induction
AT5G51990	AP2-EREBP	CBF4 / DREB1D	5.9
AT4G17880	bHLH	MYC4	5.9
AT1G22070	BZIP	TGA3	5.8
AT2G21230	bZIP		5.3
AT5G62110			5.2

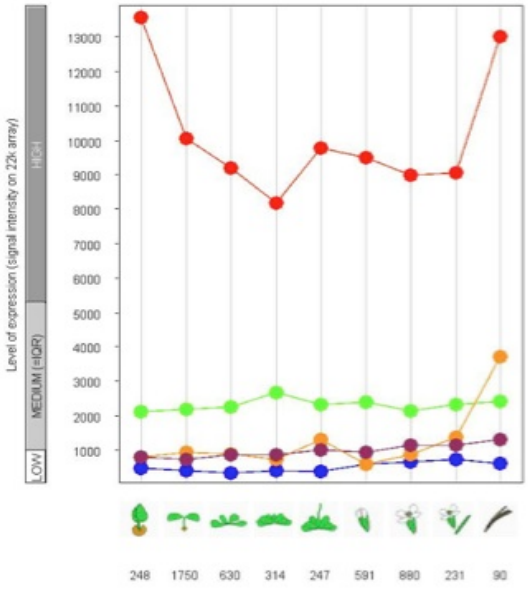


AT Code	TF Family	Name	Fold of Induction
AT2G37120	S1Fa-like		5.1
AT3G60030	SBP	SPL12	5.1
AT4G16750	ERF		5.1
AT3G53340	CCAAT	NF-YB10	5.0
AT4G24060	DOF (C2C2)		4.8

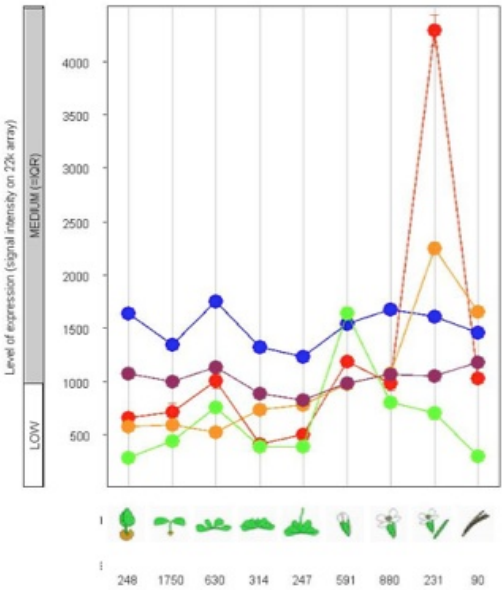


AT Code	TF Family	Name	Fold of Induction
AT4G18450	AP2-EREBP		4.8
AT4G40060	HB	ATHB16	4.7
AT3G56520			4.6
AT2G38090			4.5
AT2G04880	WRKY	WRKY1	4.5

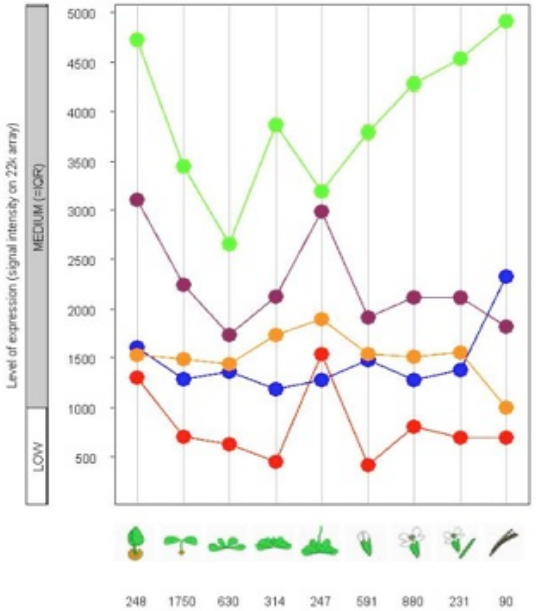
Figure 21. Genevestigator expression profiles of putative regulators of *MIR390* (Part I)
Expression profiles of remaining identified putative regulators of *MIR390*.



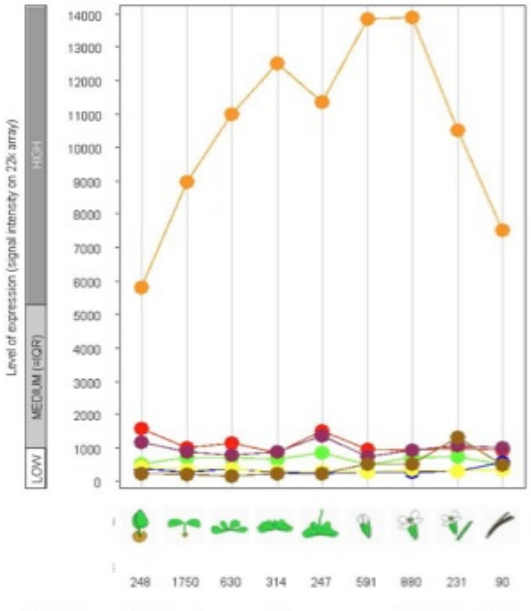
AT Code	TF Family	Name	Fold of Induction
AT4G37180	G2-like		4.5
AT1G23420	YABBY (C2C2)	INO	4.5
AT5G64270			4.5
AT1G79840	HB	GL2	4.5
AT1G66370	MYB	MYB113	4.4



AT Code	TF Family	Name	Fold of Induction
AT5G25390	ERF	SHN3	4.4
AT2G26150	HSF	HSFA2	4.4
AT3G27010	TCP	TCP20 / PCF1	4.3
AT1G20640	RWP-RK		4.3
AT4G18170	WRKY	WRKY28	4.3



AT Code	TF Family	Name	Fold of Induction
AT3G20810		JMJ30	4.3
AT4G00180	YABBY (C2C2)	YAB3	4.3
AT2G36960	MYB-related	TKI1	4.2
AT1G23380	HB	KNAT6	4.2
AT5G52010	C2H2		4.1



AT Code	TF Family	Name	Fold of Induction
AT5G06160	C2H2	ATO	4.1
AT5G18560	AP2-EREBP	PUCHI	4.1
AT2G18850	SET		4.1
AT2G46590	DOF (C2C2)	DAG2	4.1
AT5G17430	AP2-EREBP	BBM	4.1
AT1G25330	bHLH	CES	4.1
AT2G22430	HB	ATHB6	4.0

Figure 22. Genevestigator expression profiles of putative regulators of *MIR390* (Part II)
Expression profiles of remaining identified putative regulators of *MIR390*

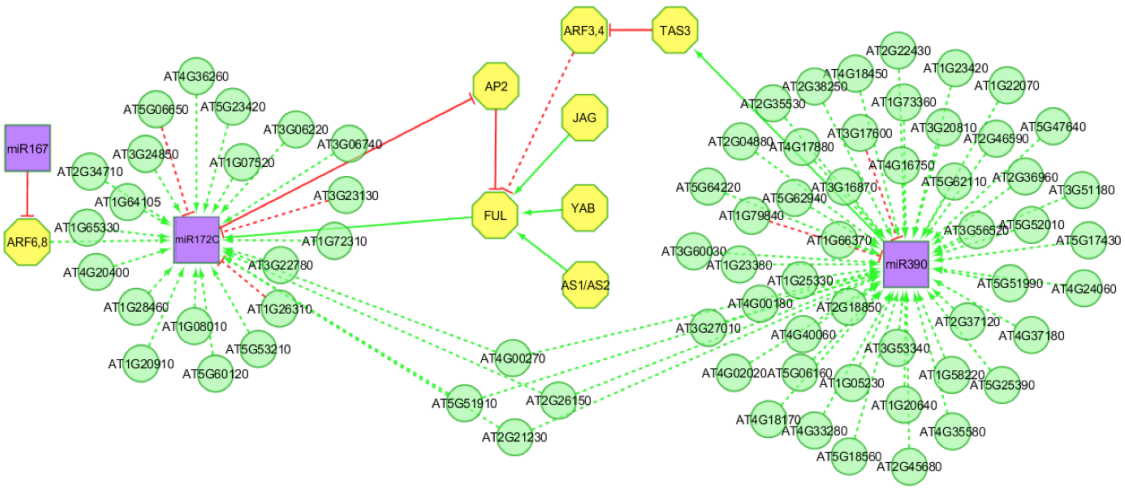


Figure 23. Revised transcriptional and post-transcriptional regulatory network orchestrating fruit development and growth

Genetic regulatory network of fruit development including the currently know players, and the putative regulators of *MIR172C* and *MIR390*

Table 1. Oligonucleotides Used to Create *MIR172C*$^{AuxRE-/-}$ Construct and *GUS* Reporter
The following oligonucleotides were used to mutagenize each of the AuxRE motifs contained with the *MIR172C* promoter. The motif being mutated is highlighted in bold and altered base pairs underlined. In the columns to the right, the WT motif sequence is given. Restriction enzymes, if any, located within the oligo are italicized and given on the right.

Oligo Name	Oligo Sequence 5' -> 3'	WT Motif Sequence 5'->3'	Restriction Enzyme
oJJR175 1st fragment	TT*GGTACC*AACTGCTATAGTAGGATCC ACATGTGC		KpnI
oJJR176 2nd fragment	TT*GTCGAC*GGTTGATGATAGGGATGTA TG		SalI
oJJR397 2nd fragment	CTTCTCCG**TATATA**ACGCT**TATATA**TT CCCTCTC	TGTCTC	
oJJR398 1st fragment	AGAGGC**TATATA**TGCGA**TATATA**AGG AGAGATGG	GAGACA	

Table 2. Oligonucleotides Used to Create MIMICRY Lines
The following oligonucleotides were used for the PCR-amplification of the *MIM172* and *MIM159* fragments. Each fragment produced contains a restriction enzyme site on either end to allow its insertion into pBJ10xOP and pGreenII0179 vectors. Restriction sites within each oligonucleotide sequence are underlined, with the corresponding restriction enzyme (RE) given in the next column.

Oligo Name	Oligo Sequence 5' -> 3'	RE	Description
oJJR267	TTGGTACCAAACACCACAAAAACAAAAG AAAAATGGCCATC	KpnI	5' border of *MIM172* and *MIM159* constructs
oJJR268	TTGGATCCAAGAGGAATTCACTATAAAGA GAATCGG	BamHI	3' border of *MIM172* and *MIM159* constructs

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