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Dissecting the Role of Small RNAs during Replum Development in the *Arabidopsis thaliana* Fruit

A Thesis submitted in partial satisfaction of the requirements
For the degree Master of Science

in

Biology

by

Ting Ting Hon

Committee in charge:

Professor Martin F. Yanofsky, Chair
Professor Nigel Crawford
Professor Jose L. Pruneda-Paz

2013
The Thesis of Ting Ting Hon is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego
2013
DEDICATION

I wish to dedicate this thesis especially to my mother, for always supporting me and truly inspiring me to be the person I am today, for her unconditional love and sacrifices. To my family, for their love and encouragement.
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ABSTRACT OF THE THESIS

Dissecting the Role of Small RNAs during Replum Development in the Arabidopsis thaliana Fruit

by

Ting Ting Hon

Master of Science in Biology

University of California, San Diego, 2013

Professor Martin F. Yanofsky, Chair

In Arabidopsis thaliana the majority of the fruit comprises an ovary with three primary tissue regions; the valve, the valve margin and the replum. Interestingly, there exists active cell proliferation in the replum domain, which leads to the formation of specialized structures located inside of the ovary, including the ovules. This feature makes the replum tissue to be considered one of the “quasi-meristem” structures present in the Arabidopsis thaliana fruit.
A set of regulatory genes has been identified to control different aspects of replum development and growth, including the replum identity genes \textit{RPL} and \textit{BP}. However, keeping in mind the meristem-like features that the replum has we wanted to explore whether additional meristem-related genes are also impinging upon replum formation.

We have uncovered an additional player participating in replum morphogenesis, which, interestingly, is post-transcriptionally regulated by small RNAs. A comprehensive and detailed analysis of this novel factor in the context of replum development, as well as the importance of its regulation by small RNA in this developmental process are the main focuses of this work.
INTRODUCTION

*Arabidopsis thaliana, a reference organism in the Plant Biology field*

Biological phenomena, such as growth or development, reflect the real complexity of living systems. To sort the complexity of these (and many others) biological puzzles a number of organisms have been chosen to examine and dissect such biological processes. Some of the most exploited examples are *Escherichia coli* (a gram negative bacterium), *Bacillus subtilis* (a gram positive bacterium) *Neurospora crassa* (orange bread mold), *Ceanorhabditis elegans* (a nematode) or *Drosophila melanogaster*. *Arabidopsis thaliana* (*Arabidopsis* hereafter), a small flowering plant in the Brassicaceae family, common food crops such as cabbage, cauliflower, radish are known as the close relatives. Thanks to its short life cycle and miniature nature, *Arabidopsis* has been considered to be the reference organism in the Plant biology field and for many years.

Since the completion of the whole genome sequencing of *Arabidopsis* in 2000, genome-wide mutagenesis has been actively performed through transfer DNA (T-DNA) insertion to screen for loss of function mutants (Alonso et al., 2003) (Fig. 1C). Together with the ease of lateral gene transfer via *Agrobacterium tumefaciens* transformation (Clough and Bent, 1998; Meyerowitz, 1989), *Arabidopsis* has been heavily used to dissect different plant developmental processes, including fruit morphogenesis, apical meristem and root development.
Vegetative development in *Arabidopsis*

During embryogenesis, plant meristematic structures are established to provide most of the post-embryonic cells that will constitute the organs of the plant throughout its life cycle. During embryogenesis two main meristems are established: an aerial meristem at the growing tip of the shoot (shoot apical meristem; SAM) and an underground meristem at the root apex (root apical meristem; RAM see later sections) (Fig 1A). These two pools of stem cells are generated post-embryonically from the stem cell niche located between two cotyledon primodia, namely the shoot apical meristem (SAM). The root apical meristem (RAM), on the other hand, is located at the tip of the growing root harboring the underground system (Aida et al., 1999; Takada et al., 2001; Hibara et al., 2003; Taoka et al., 2004; Lee et al.,2009; Girin et al., 2009) (Fig.1B). Since plants produce new organs continuously throughout their life cycle, these pluripotent cells are vital in progressing the embryo into a functional unit by going through different stages of development, proper regulation of events is of extreme importance.

Shoot Apical Meristem formation and maintenance

A number of genes are known to be expressed in the SAM, and two homeodomain transcription factors were found to play pivotal roles guarding SAM formation and maintenance. While *SHOOT MERISTEMLESS* (*STM*) is expressed throughout the SAM, preventing the initiation of cell differentiation, *WUSCHEL* (*WUS*) expression is limited to the core of the SAM (Mayer et al., 1988; Takada et al., 2001;
WUS and STM were proposed to work in two parallel pathways since STM is not required to initiate WUS expression (Mayer et al., 1998; Takada et al., 2001). To date, there are 15 members in the family of \textit{WUSCHEL}-related homeobox (WOX) genes, \textit{WUSCHEL1} (\textit{WUS1}) functions to organize stem cell in the SAM (Laux et al., 1996; Mayer et al., 1998; Shimizu et al., 2008) while other WOX genes mark cell fate decisions by preventing cell differentiation (Haecker et al., 2003; Deveaux et al., 2008; Shimizu et al., 2009). WUS expression in the SAM is limited to the central zone due to the negative regulation by \textit{CLAVATA3} (\textit{CLV3}), maintaining the stem cell pool size (Clark et al., 1995, 1996; Aida et al., 1999; Tucker and Laux, 2007) (Fig.4A).

In a parallel pathway, the organ boundary genes separate the developing cotyledons and SAM. \textit{CUC1}, \textit{CUC2} and \textit{CUC3} encode NAC domain proteins and work redundantly in cotyledon separation. They were shown to be epistatic to STM since in \textit{cuc1 cuc2} double mutant background seedlings, two cotyledons primordia are fused without the SAM and STM is not expressed (Aida et al., 1997; Aida et al., 1999; Takada et al., 2001; Taoka et al., 2004; Girin et al., 2009; Spinelli et al., 2011). After organ primordial initiation, STM and \textit{CUC} cooperate in a looped circuit which is modulated through the activity of the small RNA miR164 (Fig.4A). \textit{CUC} and miR164 are both up-regulated by STM induction, but \textit{CUC}, in turn, prevents STM ectopic expression in the SAM boundary and in the leaf primordia, while itself being down-regulated by \textit{miR164} (Spinelli et al., 2011).
On top of setting boundaries, cell fate is tightly regulated as SAM and leaf-promoting factors are mutually exclusive and repress one another in their domain of expression. The MYB domain protein ASYMMETRIC LEAVES1 (AS1) and the LATERAL ORGAN BOUNDARY (LOB) protein AS2 conform the so-called AS function, which regulates leaf initiation and differentiation (Byrne et al., 2003) and prevents the ectopic expression of meristematic genes (such as STM) in lateral organ primordia. The C2H2 Zn Finger transcription factor JAGGED (JAG) and the YABBY transcriptional regulator FILAMENTOUS FLOWER (FIL) act, in coordination with the AS function to also repress the expression of class I KNOX genes including STM. Likewise, STM keeps the stem cells undifferentiated through repression of JAG/FIL and AS1/2 (Timmermans et al., 1999; Tsiantis et al., 1999; Girin et al., 2009) (Fig. 4B).

The BEL1-like homeodomain transcription factor REPLUMLESS (RPL), is also expressed in the SAM. Its presence is critical in establishing proper phyllotaxy and internode patterns (Byrne et al., 2003; Smith and Hake, 2003; Girin et al., 2009). RPL is believed to form heterodimers with class I KNOX genes, including STM, BREVIPEDICELLUS (BP), KNOTTED IN ARABIDOPSIS THALIANA2 (KNAT2) or KNAT6 (Bellaoui et al., 2001; Smith and Hake, 2003; Girin et al., 2009).

The CLASS III HOMEODOmain-LEUCINE ZIPPER (HD-ZIP III) genes have been shown to regulate apical fate during plant embryogenesis, in SAM initiation and homeostasis and more importantly lateral organ polarity (Prigge et al., 2005; Ochando et al., 2006; Ochando et al., 2008, Smith and Long, 2010; Turchi et al., 2013). During development, class I KNOX genes and HD-ZIP III expression patterns are mutually
exclusive (Ochando et al., 2006) (Fig.4B). Interestingly, the small RNA families miR165 and miR166 post-transcriptionally regulate HD-Zip III. This regulations has been proven to be important in meristem maintainance and criitical in the regulation of abaxial-adaxial polarity of lateral organs (Prigge et al., 2005; Ochando et al., 2006; Ochando et al., 2008; Miyashima et al., 2013; Scarpella et al., 2013).

**Plant root development**

The vast majority of land plants have developed an underground root system, which is tailored to adjust to environmental cues and adapt to changing availability of water and nutrients. Although many phytohormones have been shown to be important during root development, auxin is a key player in promoting primary root growth and in initiating lateral root formation (Benkova et al., 2009). Local auxin maxima cause induction of lateral roots branching from the primary root (Dubrovsky et al., 2008).

Auxin signaling is translated into a gene expression response by a set of transcription factors called AUXIN RESPONSE FACTORS (ARFs). These transcriptional regulators bind o AuxRE (Auxin response Elements) cis-motifs present in the promoters of auxin response genes (Tiwari et al., 2007). Among other ARFs, it has been previously shown that ARF3 and ARF8 impinge upon root growth habit in Arabidopsis. Whereas, ARF8 seems to promote root growth, ARF3 inhibits lateral root formation and growth, which again show the importance of auxin in controlling root development.
The *Arabidopsis thaliana* female reproductive organ

During the floral transition plants switch from vegetative to reproductive growth, and the SAM transforms into an inflorescence meristem surrounded by floral meristems containing, each of them, a whorled of flower organ primordial (Ferrándiz et al., 1999; Huijser and Schmid, 2011). *Arabidopsis* flowers are composed of four whorls of organs, from the outermost sepals, followed by petals, stamens, to the innermost, two fused carpels that after fertilization will become the fruit (Dinneny and Yanofsky, 2005).

In nature, fruit have evolved a specialized tissue organization that facilitates fertilization, protects and nourishes seeds as they develop and ultimately assists in their dispersal. The ovary contains and protects the seeds and, on the outer side, can be anatomically divided into three main territories (Fig.2): the replum, which retains meristematic characteristics; the valves, at lateral positions and valve margins, located at the valve-replum boundaries. The septum arises from the adaxial (inner) side of the replum and divides the ovary into two chambers (locules). Inside these locules, ovules (and later the seeds) are positioned alongside the septum (Ferrándiz et al., 1999; Roeder and Yanofsky, 2006).

Multiple regulatory nodes have been reported to take place both in vegetative and reproductive growth. The carpel has long been quoted as a modified leaf because they both share basic developmental pathways and possibly have a common evolutionary origin (Roeder and Yanofsky, 2006; Ferrándiz et al., 2010). The replum has been granted the term ‘quasi meristem’ since it retains a zone of active cell proliferation that give rise to inner structures of the fruit, such as the septum and ovules (Girin et al., 2009; Gomez
et al., 2011, our unpublished data). In addition, and more interestingly, regulatory networks observed in the SAM (true meristem) are also proposed to take place in replum (Alonso-Cantabrana et al., 2007; Girin et al., 2010; Ripoll et al., 2011).

**Current regulatory network controlling *Arabidopsis* fruit patterning**

As complex as interactions taken place in the SAM, the degree of modulation in fruit patterning is beyond imagination. The Yanofsky lab and other groups have put significant effort over the years into identifying and characterizing the genes that control *Arabidopsis* fruit development. We have been able to identify a handful of transcriptional regulators orchestrating the correct regionalization and polarity of the fruit (Dinneny et al., 2004; Dinneny, et al. 2005; Roeder et al., 2006). However, although many genes have been already identified the complete picture still remains unfinished.

Different sets of regulatory genes were reported to act in distinct territories of the fruit and mutually antagonize to generate and maintain the identity of each territory (Fig.3). According to our current model, *BP* and *RPL* expressions are confined to the replum through the negative regulation of the valve, valve margin identity genes and together with the floral homeotic gene *APETALA2* (*AP2*), *AS* function and *JAG/FIL* activity (Guo et al., 2008; Ripoll et al., 2011; Semiarti et al., 2001). Valve margin identity genes, including MADS-box genes *SHATTERPROOF1* (*SHP1*), *SHATTERPROOF2* (*SHP2*) and bHLH transcription factors *ALCATRAZ* (*ALC*), *INDEHISCENT* (*IND*), control valve margin formation (Liljegren et al., 2000; Liljegren
et al., 2004; Rajani and Sundaresan, 2001). The refined expression patterns of these genes are limited to the valve margin due to the joined force from valve identity gene FRUITFUL (FUL) and RPL (Gu et al., 1998; Liljegren et al., 2004; Roeder et al., 2003; Smith and Hake, 2003) (Fig.3). The expression of FUL within the valves is achieved through interactions with AP2, AS1,2 and JAG/FIL (Alonso-Cantabrana et al., 2007; Dinneny et al., 2005; González-Reig et al., 2012). Thus in mutant affected in valve, valve margin or in ap2, as1 and as2 single mutants results in oversized repla (Alonso-Cantabrana et al., 2007; Ripoll et al., 2011) as also described for plants over-expressing BP. It has been shown that the formation of BP-RPL heterodimers is required for their nuclear localization and target gene regulation (Rutjens et al., 2009). Thus in bp rpl double mutants, fruit do not form outer replum tissues (Fig.9G).

An additional replum gene has been recently identified. The WUSCHL LIKE HOMEobox (WOX) gene WOX13, a member of the WUS family, prevents the ectopic expression of JAG/FIL activity in the replum, which in turn promotes replum development. Misexpressing WOX13 leads to an enlarged replum and valve defects reminiscent to those of fil/jag mutants (Romera-Branchat et al., 2013).

Small RNAs and plant development

The 20-24 nucleotides long small non-coding RNAs, has now became a research hotspot for many to study because of their abilities in refining gene expressions and fine-tuning the level of mRNAs, either by translational inhibition or transcript cleavage
The two classes of small RNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs), thus provide another dimension to the molecular regulatory network.

Targets of plant miRNAs can be easily predicted due to the highly complementary nature between the miRNAs and the mRNA transcript (Bartel and Bartel, 2003). It was extensively described in previous studies that miRNAs is involved in myriad developmental processes, such as leaf patterning (Mallory et al., 2004; Nikovics et al., 2006), floral identity and development (Baker et al., 2005), flowering time (Schwab et al., 2005), developmental phase transition (Schwab et al., 2005; Wu and Poethig, 2006), shoot and root development (Laufs et al., 2004; Mallory et al., 2004; Guo et al., 2005; Schwab et al., 2005; Marin et.al., 2010), and plant development in response to hormone signaling (Guo et al., 2005). To ensure the homeostasis of miRNAs, which allows proper development, the SMALL RNA DEGRADING NUCLEASE (SDN) genes encode a family of exoribonucleases to degrade mature, single-stranded miRNAs in Arabidopsis (Ramachandran and Chen, 2008b).

Trans-acting small interfering RNAs (ta-siRNAs) is a group of small interfering RNAs. While ta-siRNA and miRNA mediate similar gene repression post-transcriptionally, ta-siRNA was encoded by the TAS gene and requires miRNA for its initiation (Vazquez et al., 2004; Allen et al., 2005; Garcia, 2008).

Although significant efforts have been made to identify the biogenesis, mode of action and degradation of small RNAs, the upstream regulatory network is still highly masked. Our recent findings uncovered the role of several post-transcriptional regulatory
miRNAs (miRs) controlling different aspects of fruit. We have also discovered that during fruit morphogenesis, both transcriptional and post-transcriptional layers of regulation are tightly linked. Using these data as a platform, we are trying to unravel, bit by bit, the precursors to all the well-defined downstream events.

**Auxin and Auxin Responding Factors (ARFs)**

Auxin, or indole-3-acetic acid (IAA) is arguably one of the most important hormones throughout a plant’s life cycle. In response to high level of auxin, derepressed auxin responding factors (ARFs) binds to auxin-response elements (AuxREs) located in promoters of auxin-response genes, modulating the level of transcription (Ulmasov et al, 1999). For many years, the family of ARFs have been extensively studied and hypothesized to be essential in myriad developmental processes (Fig.5), for example in replum formation (our unpublished data) and root development (Guo et al., 2005; Gutierrez et al., 2009; Marin et.al., 2010; Tabata et al., 2010; Tian et al.,2004; Wu et al., 2006).
MATERIALS AND METHODS

Plant Materials

*Arabidopsis thaliana* Columbia (Col-0) accession was used as wild type in this study. Plant materials used in this study were: MIR164A::GUS (this work); MIR164B::GUS (this work); MIR164C<sup>e</sup>::GUS and MIR164C<sup>s</sup>::GUS (this work); CUC1::GUS, CUC2::GUS and CUC3::GUS (Wagner et al., 2006); 35S::ARF3<sup>tasiR</sup>:GR (Ripoll et al., unpublished); 35S::ARF8:GR (this work).

Cloning strategies

*CUC1::GUS, CUC2::GUS* and *CUC3::GUS* have been previously described (Wagner et al., 2006). The β-glucuronidase (GUS) reporters of MIR164 were created by amplifying the promoter regions of MIR164A (*AT2G47585*), MIR164B (*AT5G01747*) and MIR164C (*AT5G27807*), with the use of the proof-reading, high-fidelity Phusion Taq Polymerase (New England Biolabs) from Col-0 genomic DNA. The products were double digested (Table 1) and fused to the pJJGUS T-DNA vector (Ripoll et al., 2006) containing a GUS gene reporter.

To create the MIMICRY line of MIR164 (*MIM164*), primers (Table 2) were used to amplify the MIM164 sequence from the pGem-T (Promega, Inc.) precursor vector created in Weigel lab (Todesco et al., 2010), and sub-cloned into the pBJ36-10xOP vector via *KpnI* and *BamHI* sites. The 10xOPMIM164 cassette was excised with *NotI* and cloned into the pGreenII0179 T-DNA binary vector (Hellens et al., 2000).
A segment on the 3’ end of the MIR164 promoter, here termed as MIR164C\textsuperscript{R}, was amplified using primers listed in Table 3 and the proof-reading Taq Polymerase (Phusion, NEB). The resulting PCR product was cloned into the pLacZi vector (Clontech) using DNA-assembly (Gibson et al., 2009). After checking the integrity of the sequence was then linearized with NcoI and transformed into the yeast strain YM4271 (see below); pDest22 vector (Invitrogen) with a Gal4-AD-TF was transformed into Mav103 as follows.

The inducible ARF3 GR line was created by amplifying the ARF3\textsuperscript{tasiR} sequence from the pGSD42 precursor vector (our unpublished data) using primers listed in Table 5. It was sub-cloned into the pBJ36-35SGR vector via SalI and BamHI sites. The 35S::ARF3\textsuperscript{tasiR}:GR cassette was excised with NotI and cloned into the pGreenII0229 T-DNA binary vector (Hellens et al.,2000). To generate the inducible ARF8 GR line, ARF8 cDNA was amplified with primers (Table 5) and the proof-reading Taq Polymerase (Phusion, NEB), sub-cloned into the pBJ36-35SGR vector via SalI and BamHI sites. The 35S::ARF8:GR cassette was excised with NotI and cloned into the pGreenII0229 T-DNA binary vector (Hellens et al.,2000).

**Generating and isolation of Arabidopsis transgenic lines**

All of the constructs generated were transformed into *Agrobacterium tumefaciens* (AGL0 strain) with the pSOUP carrier plasmid (Hellens et al., 2000) through electroporation (Bio-Rad). We performed *in planta* transformation with wild type (Col-0) *Arabidopsis thaliana* plants following the floral dipping method (Clough and Bent, 1988). T1 *OP::MIM164, MIR164A, MIR164B, MIR164CL* and *MIR164C* transgenic plants were
isolated by sowing seeds on MS plates containing 20mg/mL Hygromicin. T1
35S::ARF3\textsuperscript{tasiR}:GR and 35S::ARF8:GR transgenic plants were isolated by sowing seeds on MS plates selected for Basta resistance (12mg/mL Glufosinate ammonium). At least 15 T1 plants were selected for following up experiments. All these lines were genotypes to detect the presence of the transgene.

**Histology and GUS staining**

Tissues were chilled with cold 90% acetone for 15 minutes on ice, followed by a 15 minute wash with DI water at room temperature, infiltrated for 5 minutes with GUS staining solution (25mM sodium phosphate; 5mM potassium ferrocyanide; 5mM potassium ferricyanide; 2mM X-Gluc; 1% Triton X-100), and incubated overnight at 37°C.

Tissues were fixed in FAA (50% ethanol, 3.7% formaldehyde, 5% acetic acid) for 2.5 hours, embedded in Paraplast Plus after going through ethanol and Histoclear series. Seedlings were fixed as above described and embedded in JB-4 resin (Polysciences, Inc.) according to manufacturer’s instruction.

**Yeast Transformation**

Competent yeast cells were prepared fresh by growing a 50mL overnight culture of the corresponding yeast strain in YPD, shaking at 30°C. 2mL of the overnight culture was transferred to 75mL of YPD and incubated at 30°C until the OD600 reached 0.4-0.6.
50mL of the new culture was then centrifuged at 1,000xg for 5 minutes, followed by three washes with sterile milli-Q water. 1.5mL of freshly made 1xTE (10 mM Tris pH 7.5, 0.05 mM EDTA) /1xLiAc (pH 7.5) was used to resuspend the cell pellet. For each transformation, 100µL of competent yeast was added to 0.1µg plasmid DNA, facilitated with 0.1mg of single stranded carrier salmon sperm DNA. Each tube was incubated at 30°C for 30 minutes with the added 0.6mL of sterile PEG (50% PEG 3,350)/1xTE/1xLiAc. 70µl of DMSO was added to each tube and mixed by gentle inversion. The tubes were placed on 42°C heating block for 15 minutes, chilled on ice for at least 2 minutes and centrifuged for 30 seconds at maximum speed. Supernatant was removed and the pellet was resuspended in 150 µl of 1xTE. The entire content was plated on SD-ura (YM4271 strain) or SD-tra (Mav103 strain) respectively. The plates were incubated for 3 days in a humidified box at 30°C. Success of the transformations was checked by colony PCR. Write the protocol you used for yeast PCR

**Yeast –one-hybrid**

Yeast-one-hybrid assays were performed as previously described (Pruneda-Paz et al., 2009).

**RNA Isolation and mRNA Measurement**

Total RNA was extracted from inflorescence tissues using NucleoSpin RNAII (MACHEREY-NAGEL GmbH & Co) following manufacture instructions.
micrograms of total RNA was used as template for cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen) kit and oligoDT primer. Quantitative PCR (qPCR) was performed as described in (Ripoll et al., 2011) using with primers listed in Table 4 and SYBR Green PCR master mix (Qiagen).

**Dex Induction Root Assay**

Seeds of the T3 segregating transgenic lines of 35S::ARF3<sup>3asiR</sup>:GR and 35S::ARF8:GR were first grown on regular MS media plates along with WT seeds as control, the 5 days old seedlings were transferred onto MS media plates containing 5mg/mL Dexamethasone (Dex) for Glucocorticoid receptor induction. Primary root length was measured and averaged from more than 10 seedlings, before and after Dex induction. Pictures were taken 72hours after the induction and the number of lateral roots were counted and averaged for each line.
RESULTS

I. 1. Tools developed to perform in-depth analysis of the roles of ARF3 and ARF8 in root development

As mentioned earlier in the introduction, auxin response factors (ARFs) have been heavily studied because of their role in the transcriptional control of auxin responsive genes in response to changes of auxin levels, and thus their importance in plant development including fruit morphogenesis. Here we have taken one step further and developed some tools to better characterize the role of some ARFs in fruit patterning (our unpublished data).

Initially identified for interacting with adaxializing and abaxializing factors in the leaf primordia polarity (Scarpella et al., 2010), *ARF3* is also shown to be active in valves, repressing the valve regulatory genes (our unpublished data). On the other hand, *ARF8*, together with *ARF6*, also regulate different aspects of fruit development including ovule development, fertilization and valve growth as our unpublished data strongly suggest (Tabata et al., 2010; Wu et al., 2006). Previous results and our own data supports that correct fruit development requires proper regulated *ARF3* and *ARF8*). An additional interesting feature is that ARF3, ARF6 and ARF8 are post-transcriptionally regulated by small RNAs. Whereas miR167 regulates ARF6 and ARF8, the tasiRNAs generated by the *miR390/TAS3* pathway regulate ARF3 (Yoon et al., 2009; Marin et al., 2010).

In this context we decided to generate glucocorticoid receptor (GR) inducible lines for *ARF3* and *ARF8* (Fig.7A) under the control of the 35S constitutive promoter to further address the role of these transcriptional regulators during plant development,
in particular in fruit morphogenesis. This system has been widely used and fruitful results were obtained in other studies (Sorefan et al., 2009). It has been previously reported that misexpression of wild-type cDNA leads to alteration in different parts of the plant. In contrast, misexpression of ARF3 wild-type cDNA seems not to alter plant development. However, when an ARF3 tasi-RNA resistant (ARF3\textsuperscript{tasiR}) version is misexpressed, plant development is drastically impaired. Thus, we decided to generate ARF3\textsuperscript{tasiR} and ARF8 GR-inducible constructs. In the Materials and Methods section we described how these transgenic lines were generated.

I.2. ARF3 and ARF8 act antagonistically in Arabidopsis root development

As mentioned above, we wanted to test the behavior of the ARF3 and ARF8 GR-inducible lines in the context of root development. For our root assays we used homozygous T3 lines for 35S::ARF3\textsuperscript{tasiR}-GR and 35S::ARF8-GR, respectively.

Seeds were germinated on MS plates and grown for 5 days after germination. At this stage, no observable phenotypic differences were seen when 35S::ARF3\textsuperscript{tasiR}-GR or 35S::ARF8-GR root system was compared to that of wild-type plants (data not shown). At this point, we transferred wild-type, 35S::ARF3\textsuperscript{tasiR}-GR or 35S::ARF8-GR to MS+Dex plates and growing them for 72 hours. After Dex induction we observed striking differences when the root system of the GR lines was compared to that of wild-type (Fig. 8A).
When the roots of induced 35S::ARF3\textsuperscript{tasiR-GR} seedlings were tested, we observed a dramatic reduction in primary root length and the lateral root emergence was retarded (measurement of lateral root length was not taken; Fig. 8). These phenotypic defects are very similar to those previously described for plants in which ARF3 function is altered. Although not shown in this work, we also observed leaf polarity defects in 35S::ARF3\textsuperscript{tasiR-GR} seedlings. This is not surprising since ARF3 plays a role in regulating abaxial and adaxial polarity and this role is tasiRNA-dependent. Moreover, the presence of these defects further support the fact that our line behaves as expected.

The Dex-induced ARF8 lines showed a net primary root growth more than that of the wild-type control, opposite to what we have described above for ARF3\textsuperscript{tasiR}. Moreover, in these seedlings the lateral root density was conspicuously higher than that of wild-type and/or 35S::ARF3\textsuperscript{tasiR-GR} induced. In summary, ARF8 overexpression lines displayed a vigorous growth in primary roots with a blooming network of well-developed lateral roots.

To more quantitatively analyze these observations, data was collected and averaged from more than 10 plants from each of the three independent transgenic lines assayed, before and after Dex induction (Fig 8B).

With the above results, we again propose that ARF3 and ARF8 have antagonistic roles during root development because of their opposite behavior in primary and lateral root growth shown in this study. Given that it was previously hypothesized by our lab that ARF8 possibly repress ARF3 in root morphogenesis, the transgenic lines we generated is working as expected. Although we had a first peek into this regulatory node
with some confirming data, at this moment, we also prepare to perform western blot to further compare protein abundance and the efficiency in these GR driver lines. Any artifacts or discrepancies will be cleared and eliminated when the blotting is done.

II. The role of small RNAs in fruit development

Being termed ‘quasi meristem’, the replum has retained partial meristematic activities that give rise to inner structures of a fruit, such as the septum and ovules (Girin et al., 2009; Gomez et al., 2011, our unpublished data). Over the years, many have studied the genetic pathways involving in fruit development and were able to identify a handful of transcriptional regulators orchestrating the correct regionalization and polarity of the fruit (Dinneny et al., 2004; Dinneny, et al. 2005; Roeder et al., 2006), yet little was known how small RNAs fit into this puzzle.

As mentioned in the Introduction, there likely exist two parallel regulatory pathways governing replum formation. One of them involves BP and RPL (Fig. 3), whose activities, are therefore circumscribe to replum tissues (Fig.10D-E). RPL and BP are required to limit the expression of the valve margin identity genes to the valve margin (Roeder et al., 2003; Liljegren et al., 2004; Girin et al., 2011). Although in bp mutants no replum defects are reported (Fig. 9E), loss of function in BP enhances the replumless phenotype observed in rpl single mutant and both inner and outer replum domains reduced their sizes, being more dramatic than the loss of outer (abaxial) tissue.
In *bp rpl* fruit, the abaxial region of the replum is absent, whereas the adaxial (inner) domain, that contains vascular tissues, develops similar to that of wild-type fruits (Fig. 9G) (Ripoll et al., 2011). Lack of repression from *AS1*, *as1* single mutants (Fig. 9H) results in oversized replum, echoes the morphology of over-expressing *BP* (Alonso-Cantabrana et al., 2007; Ripoll et al., 2011). These results suggest that both BP and RPL are critical in the formation of the outer replum domain.

Although the fruit patterning regulatory network has been extensively examined, the layer of small RNA regulations is still far from being well-defined. Recent projects in our lab have identified several small RNAs involved in different aspects of valve development (our unpublished data). However, no replum-related small RNA was reported. As a part of our effort to delineate the role of small RNAs in fruit, we decided to search in public gene expression database and in the published literature aiming to identify a replum-related miR. As presented next, the miR164 and its targets seemed to be our best candidates.

II. 1. *CUC* miR164-regulation protects replum development

As mentioned previously, in the SAM miR164 negatively regulates *CUC* genes at the post-transcriptional level (Fig. 3 and 4), which in turn, repress the KNOX genes at the boundaries of the SAM and leaf primordia (Spinelli et al., 2011), similarly as *ASI/AS2* do (Fig. 4B) (Timmermans et al., 1999; Tsiantis et al., 1999; Girin et al., 2009). Interestingly,
KNOX genes are also negatively regulated by the AS function in fruits (Alonso-Cantabrana et al., 2007).

We wanted to confirm how similar was the size of the SAM of 6 days old in \textit{as1} and \textit{cuc2-d} (miR164-immune version of \textit{CUC2}) (Fig. 9B-C). As expected, the SAM in \textit{as1} mutant (Fig. 9B) has a deformed shape, significantly enlarged with more cell counts, longitudinally and horizontally, compare to WT (Fig. 9A) due to the loss of repression of \textit{STM} by \textit{AS1}. The SAM size in \textit{cuc2-d} (Fig. 9C) seems to be in between that of the WT and \textit{as1} mutant. \textit{cuc2-d} has the same number of layer of cells longitudinally as WT, but slightly wider SAM due to the mis-regulated boundary region between the SAM and leaf primodia.

With the confirmed interaction between miR164 and \textit{CUC} in the SAM, it was tempting for us to believe that this layer of regulation is conserved between SAM and fruit replum. As we observed and as reported in previous studies, plants harboring a miR164-immune version of \textit{CUC2} (\textit{cuc2-d}) generated vegetative mutant phenotypes similar to those in \textit{BP} and/or \textit{RPL} genes are mis-expressed (Laure et al., 2009) or \textit{as1} (Fig. 9C). We thus decided to take a closer look at the fruits of these plants. We found that, whereas in wild-type fruit both inner and outer replum domains are well established, in \textit{cuc2-d} siliques the outer domain was replaced by inner structures (vascular tissue, septum, placenta) (Fig.9J). These defects are very similar to those described before for other mutant backgrounds with altered inner (adaxial)/outer (abaxial) polarity such as \textit{incurvata4-d} (Ochando, I. et al., 2006). We observed that \textit{miR164a,b,c} triple mutant individual showed similar fruit defects as those described for \textit{cuc2-d} (Fig.9I). Taken
together, these data suggest that misexpression of *CUC2* has a deleterious effect on replum polarity and, further, that the correct specification of inner-outer replum requires miR164 function.

II. 2. Mapping and analyzing the expression patterns of *CUC* and *MIR164* genes in the *Arabidopsis thaliana* fruit

Our next step was to analyze the expression of the *CUC* boundary gene in fruits using the β-glucuronidase (*GUS*) reporter lines *CUC1::GUS*, *CUC2::GUS* and *CUC3::GUS* respectively. *CUC1::GUS* is active at low levels in the abscission region. *CUC2::GUS* and *CUC3::GUS* display patches of expression in the valves and in the valve-replum boundaries (Fig. 10F-H), which likely is the valve margin. *GUS* reporter signal for *CUC2* is present in the inner tissues of the fruit, including the adaxial (inner) region of the replum and placenta and ovules (Fig.10I). However, no activity was detected in replum tissue. We therefore, decided to identify if any of the *MIR164* genes was expressed in replum tissue.

There are a total of three encoding-loci for miR164 (*MIR164A*, *B* and *C* respectively). To examine the *MIR* expression patterns, we generated transgenic lines with *GUS* reporter for each of the three encoding-locus (see material and methods). This allowed us to generate a map of *MIR164* expression pattern. We decided to take a closer look at our *GUS*-reporters in stage 13-14 pistils.
*MIR164A::GUS, MIR164B::GUS* and *MIR164C::GUS* showed distinct domains of expressions. *MIR164A::GUS* constructs displayed low level of expression in the style and the upper portion of the valve (Fig.10A). *MIR164B::GUS* construct showed high level of activity in the style, valves and replum, and no activity in the valve margins (Fig.10B). *MIR164C::GUS* constructs exhibits refined expression only in the style and replum (Fig.10C). *MIR164C::GUS* was active in replum domain mirroring that of the previously published transgenic line *MIR164C::GFP* (Green fluorescence protein) (Sieber et al., 2007). Whereas at early stages of development, reporter signal is present throughout the entire replum, as fruit development proceeds, it becomes restricted to the outer replum (Fig.10J).

**II. 3. BP and RPL are possible upstream regulators of miR164**

As part of the general effort that our lab is developing to uncover the upstream regulators of small RNA-encoding genes, we more carefully studied the regulatory regions of the replum-specific miR164 encoding gene *MIR164C*.

Initially we generated a truncated version of *MIR164C::GUS* in which a 1,000 bp portion in the 5’ region of the *MIR164C* promoter was deleted. We termed this construct as *MIR164C*:GUS. To our surprise, no replum signal was detected when GUS expression was assayed in *MIR164C*:GUS (Fig. 11), suggesting that this portion of the promoter somehow is required for MIR164C replum activity. We thus termed this MIR164C promoter region “RRR” (Replum Regulatory Region; Fig. 11).
We then *in silico* scanned the RRR domain and found, among others, two putative cis-regulatory motifs for BELL homeodomian transcription factors (Figure 11) (Luo H, Song F, Goodman RM, Zheng Z.), motifs that can be recognized by BELL transcriptional regulators like RPL. The fact that, on one hand, the transgenic reporters for *MIR164C, BP* and *RPL* genes strikingly overlapped in replum tissues (Fig.10C-E) and, on the other hand, that we identified putative cis-motifs for RPL; made us wondering whether *BP/RPL* might be regulating *MIR164C* in replum tissues.

At this point, we decided to assay *MIR164C::GUS* activity in *bp, rpl* and *as1* mutant backgrounds. To speed-up this set of experiments, we transformed into these backgrounds *MIR164C::GUS* construct. We successfully obtained T1 transgenics in *bp* and *as1* and compared the resulting expression patterns to those in WT seedlings and fruits. In *bp* mutant *MIR164C::GUS* expression has decreased in the leaves and also the primary root (Fig.13B). Similar changes in activity of the *GUS*-reporter are also detected in the stage 13 inflorescence medial tissues (Fig. 13E). In contrary, the *GUS*-reporter activity is significantly increased in the *as1* mutant seedling and fruits (Fig.13C,E). Since *AS1* is known to repress *BP* and *RPL* expression in the shoot apex (Timmermans et al., 1999; Tsiantis et al., 1999; Girin et al., 2009), gain of *MIR164C* reporter activity is likely due to an increased *BP/RPL* expression in the loss of function *as1*.

We plan to further confirm and quantify the expression of *MIR164C* in effect of a knockout of possible upstream regulators. As an initial step towards this direction, we performed real-time PCR to study the relative expression of *MIR164C* in WT versus *rpl* single mutant. Results are as predicted that a conspicuous decrease of *MIR164C*
expression is observed (Fig.12). Once again, all the data together supports the hypothesis that \textit{BP/RPL} is acting upstream of \textit{MIR164C} in a direct or indirect manner.

II. 4. Testing possible interaction between the replum identity genes \textit{RPL} and \textit{BP} with the \textit{MIR164C} promoter region

To further substantiate our model in which \textit{BP} and \textit{RPL} regulate (likely directly) the expression of \textit{MIR164C} in the replum through their binding to the promoter region, termed earlier as \textit{MIR164C} \textit{R} (Fig. 11) we decided to employ the yeast one-hybrid (Y1H) system to detect any possible \textit{in vivo} interaction (Pruneda-Paz et al., 2009).

As described previously in materials and methods, we generated a construct with the \textit{MIR164C} \textit{R} promoter region was cloned into the pLacZi (Clontech) binary vector, controlling the expression of the LacZ reporter gene. We also created \textit{pDest22BP} and \textit{pDest22RPL} following a Gateway-based approach. In these constructs, RPL and BP were respectively cloned in frame with the activation domain (AD) present in pDest22. After checking the integrity of these constructs by sequencing, we transformed with the corresponding pLacZi and pDest22 into yeast strain YM4271 and Mav103 respectively. After mating, promoter binding of the specific transcription factor will activate a downstream reporter gene (\(\beta\)-galactosidase, \textit{LacZ}) and we reasoned that there are three possible scenarios (Fig. 14). BP and RPL are reported in different sources to function as heterodimers (Bellaoui et al., 2001; Byrne et al., 2003; Smith et al., 2002; Kanrar et al., 2006; Rutjens et al., 2009), we would expect scenario 1 and 2 not going to drive the
transcription, if any, of *MIR164C*. Unfortunately, at this moment we are still trouble shooting to succeed in transforming the constructs pLacZi *MIR164C*\(^R\) and pDest22RPL into yeast, however the mating and the following \(\beta\)-galactosidase assay will be done as soon as possible.

### II. 5. Approach to impair miR164 function

To better understand the role of miR164 in replum development, we decided to impair miR164 function by generating artificial target mimics as stated in materials and methods. The target MIMICRY (MIM) technology was used to sequester in vivo the miRNA, miR164 in this case, decaying its activity (Todesco et al., 2010). T1 transgenic inducible line of *OP::MIM164* exhibited partially serrated leaves, with serration in only the basal region of the leaf (Fig15A). This serration pattern is also comparable with that of *miR164a,b,c* triple mutant plant. On top of that, we also observed ectopic tissue growth from the valve margin, which is also seen in *cuc2-d* mutants (Fig. 15B). This phenotype is possibly due to the fact a carpel developmental defect suggested by previous studies (Laure et al., 2009; Todesco et al., 2010). These results once again stressed on the importance of miR164 regulation of *CUC* in the replum tissue.

Currently in the lab, we made a driver line in which the *LhG4* transgene was under the control of the *BP* promoter (*BP::LhG4*), which will soon be ready to cross to the mimicry line *OP::MIM164*. It is a versatile way to deliver inducible tissue-specific gene expression (Baroux et al., 2005; Brand et al., 2006), so the mutant phenotype in *OP::MIM164* will be restricted to tissues or cell types where *BP* is expressed. After all
we can compare the F1 phenotype to the control (OP::MIM164). As of now, we are unable to report any findings using this technique, but hopefully this piece of information will be obtained soon.
DISCUSSION

I. Tools developed to further analyze the role of ARF3 and ARF8 in plant development

We have generated two transgenic Dex-inducible gain-of-function lines for ARF3 and ARF8, two of the Auxin Response factors in the Arabidopsis genome. By different techniques we have determined that these two lines accumulate high levels of mRNA and protein respectively. These molecular data strongly suggest that both ARF3-GR and ARF8-GR might behave as gain-of-function backgrounds. Thus, to one step further and evidence at the phenotypic level our molecular results.

It has been proposed that ARF3 and ARF8 work antagonistically in different developmental pathways in Arabidopsis including root development (our unpublished data and Yoon et al., 2009; Marin et al., 2010; Gutierrez et al., 2009). We then decided to test whether our lines behaved as previously proposed. No evident root defect was detected in neither ARF3-GR nor ARF8-GR without Dex treatment, these same lines treated with Dex show dramatic phenotypes.

Whereas induction of ARF3 drastically impaired primary root growth and lateral root formation comparing to wild-type, in ARF8 dex-treated plants root growth was overall induced. These opposite phenotypes are in line with previous reported data, which again validate our transgenic lines.

After checking the protein abundance with western blot, we believe that these lines can also be used as tools to perform chromatin immune-precipitation (ChIP). As
ARF3 and ARF8 were shown to not only participate in root development, but also fruit patterning, one of our goals is to identity the possible downstream targets of ARF3 and ARF8 in different developmental processes, especially fruit morphogenesis. The data will get cooperated into other ongoing projects in the lab, further expanding the current genetic network controlling fruit development in *Arabidopsis*, and also apply this understanding to other food-related crops.

II.1. miR164 limits the expression and activity of CUC in the SAM and fruit replum

Previous work have extensively studied the role of *CUP-SHAPED COTYLEDON* (*CUC*) in setting boundaries in the SAM, not only initiates *SHOOT MERISTEMLESS* (*STM*) which is required for SAM formation, but also separates the developing SAM and cotyledons, preventing the fusion of cotyledon primordial. (Aida et al., 1997; Aida et al., 1999; Takada et al., 2001; Taoka et al., 2004; Girin et al., 2009; Spinelli et al., 2011). *CUC*, however, is being down-regulated by miR164 (Spinelli et al., 2011) (Fig.4A).

After carefully studying the *GUS* reporter activities of *MIR164* (*MIR164A, MIR164B, MIR164C*) and *CUC* genes (*CUC1, CUC2, CUC3*) in the fruit, we noticed their expression patterns do not overlap (Fig.10A-C, F-J). To be more specific, whereas, for example, *MIR164C* is expressed in the replum, *CUC2* is not (Fig. 10C, G, I and J). We then proposed that miR164 is likely preventing the ectopic expression of *CUC* genes in replum tissues.
II.2. Two approaches used to dissect the importance of $MIR164$ and $CUC$ in fruit development

In order to further analyze the roles of $MIR164$ and $CUC$ in fruit development, we first compared the replum phenotype of $miR164a,b,c$ triple mutant and the $cuc2-d$ mutant to the WT (Fig. 9D,I,J). $miR164a,b,c$ triple mutant has impaired microRNA activities, whereas a dominant allele for $CUC2$, $cuc2-d$ has a nucleotide change in the miR164 binding site, which makes the transcripts of this allele insensitive to the regulation of miR164. Strikingly, both mutants show a polarity defect that the inner replum expands in expenses of the outer replum. Ectopic tissues are also observed growing out from the replum in both cases. This observation further fuels the idea that miR164 is important in replum development.

Another way that we use to study the regulation of miR164 is to create target mimics, mimicry technology uses an artificial nucleic acid sequence that sequesters a particular miR, causes a drastic reduction in its function. In this work, we generated $MIM164$ transgenic lines which sequester specifically miR164 and lower its activity. In the $MIM164$, we observed similar ectopic tissue growth also seen in $cuc2-d$ and $miR164a,b,c$ triple mutants. This phenotype is possibly due to a carpel developmental defect suggested by previous studies (Laure et al., 2009; Todesco et al., 2010). $MIM164$ transgenic lines also exhibits partially serrated leaves, the serration pattern is also comparable with $cuc2-d$ and $miR164a,b,c$ triple mutants (Fig. 15B,C,D). Again we showed that $MIR164$ and $CUC$ are crucial in proper replum development.
II.3. *RPL* and *BP* are possible upstream regulators of *MIR164C* in the replum

The combined activities of the homeodomain transcription factors *REPLUMLESS* (*RPL*) and *BREVIPEDICELLUS* (*BP*) is key for the correct specification of the outer (or abaxial) replum (Roeder et al., 2003; Alonso-Cantabrana et al., 2007; Ripoll et al., 2011; Romera-Branchat et al., 2013). Thus in *bp rpl* double mutants, fruit do not form replum tissues (Ripoll et al., 2011). It has been shown that the formation of BP-RPL heterodimers is required for their nuclear localization and target gene regulation (Bellaoui et al., 2001; Byrne et al., 2003; Smith et al., 2002; Kanrar et al., 2006; Rutjens et al., 2009).

In this study we have provided strong evidence on the more than possible role for BP-RPL heterodimers in regulating *MIR164C* gene expression in the replum, regulatory regions most likely through the BELL homeodomain transcription factor binding site cis motifs localized within the *MIR164*^R^ promoter fragment (Fig.11). This conclusion is supported by the fact that *MIR164C::GUS* activity is drastically lowered in *bp* whereas up-regulated in *as1* mutant backgrounds, in which fruits replum identity genes are misregulated. Thus, since *AS1* is known to be upstream regulator of *BP* and *RPL* in fruit development (Guo et al., 2008; Ripoll et al., 2011; Semiarti et al., 2001), gain of *MIR164C* reporter activity is likely due to an increased *BP/RPL* expression in the loss of function *as1*.

On top of that, we also tried to quantitatively analyze the expression level of *MIR164C* in WT and *rpl* mutant inflorescence tissues with real-time PCR, it is not surprising that the relative expression of *MIR164C* in *rpl* mutant is dramatically reduced
comparing to WT. To further characterize the MIR164C expression qRT-PCR studies should be done in bp, bp rpl, cuc2-d and as1. We believe this will be helpful to further support our hypothesis.

As of now, although no conclusion can be drawn from the Y1H assay and more qPCR data needs to be obtained, but with all the preliminary data that we have gathered, BP and RPL seems to be directly or indirectly regulating MIR164C in the fruit.

II.4. BP/RPL-miR164-CUC regulatory module likely governs proper replum development and polarity

Although we are aware that more experiments have to be done, in this study we have studied the expression of CUC genes in fruit, we have also monitored the expression of MIR164C in the replum, which most likely to prevents CUC activity in the medial tissues. Additionally, we also showed that MIR164 and CUC have pivotal role in maintaining replum polarity. Nevertheless our results evidence that the replum identity genes BP and RPL are likely to either directly or indirectly control the expression of MIR164 in the replum. With the identification of another possible layer of regulation to properly pattern the fruit, we hope to convince that the BP/RPL-miR164-CUC regulatory module is required for proper replum development.
III. Small RNA regulation is an important aspect to study developmental processes

We have demonstrated the importance of post-transcriptional regulation via small RNA plays critical roles in fruit development. Our results, and results from others show that small RNAs regulate a wide range of targets involved in multiple aspects of plant development. They can regulate boundary identity genes, for example CUC, to auxin response factors, for example ARF3 and ARF8. The specific expression of the vast majority of small RNA encoding genes in maintaining the homeostasis of regulatory pathways to keep under control in varies developmental processes. Hopefully this work is helping to appreciate how important small RNAs are in plant morphogenesis.

In this study we have explored the role of novel regulatory genes modulating fruit development, patterning and polarity. In the long run, these investigations will help us to better understand how the fruit is sculpted in Arabidopsis. As it has been demonstrated for many other processes, this knowledge can be rapidly applied to improve different traits of crop species.

IV. Future steps

Although some promising data was obtained, this project is far from completion with many other perspectives need to be addressed and solved. First of all, we want to finish the Y1H assay as soon as possible to test direct interactions between the replum identity genes and the promoter region of our interest, MIR164C. Nonetheless, we want to elucidate if the two currently known regulatory nodes, BP/RPL and WOX13 pathways have a point of convergence in replum development. The findings in this work will hopefully serve as stepping stone to future studies.
APPENDIX A: FIGURES AND FIGURE LEGENDS

Figure 1. Anatomy of *Arabidopsis thaliana*

(A) A WT reproductive adult plant bearing inflorescence. (B) The root structure of a ten-day old seedling grown in continuous light condition. (C) Combining classical approach approach and next generation technology to study fruit development in *Arabidopsis.*
Figure 2. Anatomy of the *Arabidopsis* fruit.
The three main territories are the replum (blue), the valves (green) and the valve margins (pale pink). The valve margins are subdivided into the separation layer (purple) and the lignified layer (dark pink). See axis in arrows.
Figure 3. Current regulatory network controlling fruit patterning
The model shows the genetic interactions between regulatory genes that take place during fruit morphogenesis in Arabidopsis.
Figure 4. Genetic and molecular pathways in SAM formation and Maintenance
(A) Key players controlling SAM development and the proposed miR164-CUC circuit. (B) Key players controlling leaf development and polarity involving HD-ZIPIII.
The SAM is defined in blue and CUC activity in yellow. The abaxial side of the leaf primordium is presented in dark pink, adaxial side in light pink.
Figure 4. Continued.
Figure 5. Small RNAs biogenesis pathways, mode of action and degradation. Current model showing microRNA (miRNA) and trans-acting small interfering RNAs (ta-siRNAs) generation, degradation and how target sequences are regulated (modified from Bailey, 2012).
Figure 6. The importance of auxin in plant development.
Diagram presentation showing some developmental events in which the plant phytohormone auxin is involved.
Figure 7. Schematic presentation of the GR inducible system and proposed model for ARF3/ARF8 functions in root development.

(A) The schematic presentation glucocorticoid-mediated transcriptional induction system. (B) The regulatory node of small RNAs, ARF3 and ARF8 in root morphogenesis.
Figure 8. Primary root growth and lateral root development in response to dexamethasone induction in transgenic ARF3 and ARF8 overexpression lines.

(A) Comparison between root architecture of 8-d-old seedlings of the wild type, an overexpression ARF3 ta-siRNA resistant line, and an overexpression ARF8 line. (B) Dex induced changes in primary root length (C) Measurement of the lateral root density after 72 hours Dex induction. Scale bar to 10mm.
Figure 8. Continued.
Figure 9. The role of miR164 in SAM and replum development.
Toluidine Blue stained longitudinal sections of 6 days old seedlings showing the SAM of (A) WT (B) as1 and (C) cuc2-d (miR164-resistant). Toluidine Blue stained cross sections showing the replum of (D) WT, (E) bp single mutant (F) rpl single mutant (G) bp rpl double mutant (H) as1 single mutant (I) miR164a,b,c triple mutant and (J) cuc2-d (miR164-resistant) fruits.
r: replum
All scale bars to 100µM. (D to H modified from Ripoll et al., 2011)
Figure 10. Expression pattern of MIR164, CUC, BP and RPL in fruit.
Whole mount GUS reporter activities of (A) MIR164A (B) MIR164B (C) MIR164C (D) BP (E) RPL (F) CUC1 (G) CUC2 (H) CUC3 (I) Translational GUS reporter for CUC2 (J) MIR164C::GFP.
All scale bars to 1mm. (D-E modified from Ripoll et al., 2011, J modified from Sieber et al., 2007)
Figure 11. In silico scanning of the MIR164C promoter region to identify putative cis-regulatory motifs.
Figure 12. Real-time PCR quantification of the expression levels of *MIR164C* in wild-type and *rpl-2* mutant background inflorescence.
Figure 13. Comparison of miR164C expression pattern in WT and mutant background seedlings.

Whole mount GUS staining of MIR164C reporter in 7 days old seedling in (A) WT background, 10 days old seedling in (B) bp-9 mutant background and (C) as-1 mutant background. Whole mount GUS staining of MIR164C reporter in stage 13 (D) WT, (E) bp mutant background and (F) asl mutant background fruits. All scale bars to 1mm.
Figure 14. Identification of upstream regulators of *MIR164C* using the yeast-1-hybrid system.
Figure 15. MIMICRY technologies used to impair miR164 function.

20 days old seedling of (A) WT (B) OPMIM::164 (C) miR164a,b,c tm and (D) cue2-d. Fruit phenotype of (E) OPMIM::164 and (F) cue2-d. (G) a way to deliver inducible tissue-specific gene expression.

Scale bars A-D to 5mm, E-F to 1mm.
Figure 16. Proposed regulatory network controlling fruit patterning involving miR164.
Table 1. Oligonucleotides used to create GUS reporter constructs.
The oligonucleotides (primers) listed were used to amplify the putative 5’ promoter regions of the MIR164A, MIR164B, MIR164C genes and create restriction sites for cloning into the vector pJJGUS for generating GUS reporter lines. Underlined are the restriction sites used for cloning.

R.E. = Restriction Enzyme

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length (bp)</th>
<th>5’ Primer (5’→3’)</th>
<th>3’ Primer (5’→3’)</th>
<th>5’ R.E.</th>
<th>3’ R.E.</th>
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<tbody>
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<td>MIR164A</td>
<td>2112</td>
<td>oJJR362 TTGGTACCCAGATGCTC</td>
<td>oJJR363 TTGTCCGACACATGGAGAT</td>
<td>KpnI</td>
<td>SalI</td>
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<tr>
<td>(AT2G47585)</td>
<td></td>
<td>ATCACGTATGCCAAGA AATAG</td>
<td>TCTCACCCGCATTTCACAAG GC</td>
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<td></td>
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<tr>
<td>MIR164B</td>
<td>2504</td>
<td>oJJR364 TTGGTACCCAGAATAA</td>
<td>oJJR365 TTGTCCGACTCTTGCTCATC</td>
<td>KpnI</td>
<td>SalI</td>
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<tr>
<td>(AT5G01747)</td>
<td></td>
<td>TAGGAAGAACAGTAAC ATTG</td>
<td>ACACACCTTCATCATTCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIR164C</td>
<td>1817</td>
<td>oJJR366 TTGGGCCCCAAAGTCT</td>
<td>oJJR367 TTGTCCGACTTACTCACCC</td>
<td>ApaI</td>
<td>SalI</td>
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<tr>
<td>(AT5G27807)</td>
<td></td>
<td>CTGTCCTCTCTATCTTTG GTGA</td>
<td>ATTACTTTCTCTCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIR164C</td>
<td>810</td>
<td>oJJR368 TTGGTACCCAAACTGAC</td>
<td>oJJR367 TTGTCCGACTTACTCACCC</td>
<td>KpnI</td>
<td>SalI</td>
</tr>
<tr>
<td>(AT5G27807)</td>
<td></td>
<td>CCAAACTCATCACCTA TCTTTACTG</td>
<td>ATTACTTTCTCTCTTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table 2. Oligonucleotides used to amplify MIM164 construct.**
The oligonucleotides (primers) listed were used to amplify the MIM164 from the pGemT vector and clone into pJB36 10xOP.

R.E.= Restriction Enzyme

<table>
<thead>
<tr>
<th>Length (bp)</th>
<th>5’ Primer (5’→3’)</th>
<th>3’ Primer (5’→3’)</th>
<th>5’ R.E.</th>
<th>3’ R.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>541</td>
<td>oJIR267 TTGGTACCAAACACCACAA AAACAAAGAAAAATGCCC ATC</td>
<td>oJIR268 TTGGATCCAAGAGGAATTC ACTATAAGAGAATCGG</td>
<td>KpnI</td>
<td>Bam HI</td>
</tr>
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Table 3. Oligonucleotides used to create pLacZi constructs.
The oligonucleotides (primers) listed were used to amplify a region of the putative 5' promoter region of MIR164C gene (AT5G27807) and create restriction sites for cloning into the binary vector pLacZi. Underlined are the restriction sites used for cloning.

R.E.= Restriction Enzyme

<table>
<thead>
<tr>
<th>Promoter region</th>
<th>Length (bp)</th>
<th>5’ Primer (5’-3’)</th>
<th>3’ Primer (5’-3’)</th>
<th>5’ R.E.</th>
<th>3’ R.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIR164C&lt;sup&gt;R&lt;/sup&gt;</td>
<td>1007</td>
<td>oJJR366 TTGGGCCCCAAAGTCT CTGTCTCTCTATCTTTG GTGA</td>
<td>oJJR440 TTGTCCGACCAGTAAAG ATAGGTGATGAGTTTG GGTCAG</td>
<td>Apal</td>
<td>SalI</td>
</tr>
</tbody>
</table>
Table 4. Oligonucleotides used in qPCR

<table>
<thead>
<tr>
<th></th>
<th>Length (bp)</th>
<th>5’ Primer (5’ → 3’)</th>
<th>3’ Primer (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIR164 C</td>
<td>114</td>
<td>oCH11 AATGGGTGAGTAACACTT G</td>
<td>oCH12 AGAGACACGTTGGAG</td>
</tr>
</tbody>
</table>
Table 5. Oligonucleotides used to create 35S::ARF3\textsuperscript{tasiR} GR and 35S::ARF8 GR constructs.
The oligonucleotides (primers) listed were used to amplify the ARF3 and ARF8 cDNA and clone into pJB36-35SGR. Underlined are the restriction sites used for cloning.

R.E. = Restriction Enzyme

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size (bp)</th>
<th>5’ Primer (5’→3’)</th>
<th>3’ Primer (5’→3’)</th>
<th>5’ R.E.</th>
<th>3’ R.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF3\textsuperscript{tasiR}</td>
<td>~2400</td>
<td>oJJR606 AATTACTATTTACA ATGTCGACATGG TGGTTTAATCGATC TGAACGTGATGG</td>
<td>oJJR607 cagcagcagctctagaG GATCCAGAGCAATG TCTAGCAACATGTC</td>
<td>SalI</td>
<td>BamHI</td>
</tr>
<tr>
<td>ARF8</td>
<td>~2800</td>
<td>oJJR451 TTGTCACTGATGAAG CTGTCAACATCTGG ATTGG</td>
<td>oJJR452 TTGGATCCAGATGG GTCGGGTTTTGCG</td>
<td>SalI</td>
<td>BamHI</td>
</tr>
</tbody>
</table>
REFERENCES


Clark, S. E., Running, M. P. and Meyerowitz, E. M. 1995. CLAVATA3 is a specific regulator of shoot and floral meristem development affecting the same processes as CLAVATA1. *Development* 121, 2057-2067.


