UNIVERSITY OF CALIFORNIA SAN DIEGO

Adapting CRISPR/Cas9 Gene Editing to Characterize Gene Functions During *Arabidopsis thaliana* Fruit Development

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by

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The Thesis of Quoc Hung Tran is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I would like to dedicate this thesis to my parents, my wife Nicole Tran, my dog Zeeva, and my two cats Tails and Stumps, without whose support and unconditional love I would never have been able to get this far.

TABLE OF CONTENTS

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ABSTRACT OF THE THESIS

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The fruit is arguably one of the most complex plant structures, consisting of numerous distinct tissue types and where many morphogenetic events take

place in an exquisitely harmonized manner. This organ protects and nourishes the developing ovules and seeds, ensuring and providing the means for their dispersal. Moreover, fruits are the harvested products for many food crop plant species, playing a central role in agriculture. For decades, the fruit of *Arabidopsis thaliana* (a *Brassicaceae*) has been used as reference to dissect different developmental programs during fruit morphogenesis. The *Arabidopsis thaliana* fruit displays three major tissue domains easily distinguished from the outside: the valves, the valve margins and the replum. The formation of these tissues is mediated by a sophisticated network of regulatory genes, and recent studies have shown that microRNAs play crucial roles in this process. However, the lack of loss-of-function mutants in miRNA-encoding genes represented a challenge to certainly assign their functions in the fruit.

On the other hand, whereas a wealth of information has been accumulated over the years on the roles of small regulatory peptides during plant development, our knowledge on how they may impact fruit morphogenesis is very limited.

In this context, we decided to implement a CRISPR/Cas9 genome editing approach to interrogate the functions of genes encoding for miRNA and small signaling peptides, hoping to further broaden our current understanding of fruit development.

INTRODUCTION

The use of *Arabidopsis thaliana* as a model organism in Plant Biology

Arabidopsis thaliana (referred to as *Arabidopsis* from this point and on) is an angiosperm from the Brassicaceae family, the members of which include food crops such as canola, bok choy, or wasabi. As a model organism, *Arabidopsis* contains many features that allow for the ease of genetic experimentation. The small sizes of the mature plant (approximately 30 cm) and seeds allow for the storage of large populations of plants and seed offspring (50,000 seeds in a standard 1.5 mL microfuge tube), while a short life cycle of 5-6 weeks allows for rapid population propagation. An additional feature that also facilitates genetic strategies is the relative ease in cross-fertilization between mutant lines. *Arabidopsis* has a small genome made up of approximately 125 Mb (megabases) and its haploid genome contains five chromosomes (Meyerowitz, 1989).

Perhaps one of the most fundamental features that certainly expediated research in Arabidopsis was the efficiency by which transgenesis can be achieved using *Agrobacterium* (Alonso et al., 2003). This Gram-negative soil bacterium contains the plasmid Ti capable of being integrated into the genome of *Arabidopsis,* acting as a vector for the delivery of modified genetic material. In 2000, the *Arabidopsis* genome was completely sequenced, which certainly has enabled us to further expedite our research. (Arabidopsis Genome, 2000). The

combined characteristics of the plant and the various molecular biology methodologies developed have cemented *Arabidopsis* as a model organism.

The vegetative development of *Arabidopsis*

During Arabidopsis embryogenesis, a rudiment of the mature plant is developed. At this stage, localization of meristematic structures establishes the Root and Shoot Apical Meristems (RAM and SAM, respectively) (Huijser and Schmid, 2011). These meristems act as pluripotent stem cell niches, which will give rise to all post-embryonic organs. The underground RAM eventually develops into roots, and the SAM develops into other structures aerial structures (except the hypocotyl and cotyledons, as they form during embryogenesis) (Figure 1).

There are two important vegetative developmental stages: juvenile and adult. In *Arabidopsis*, juvenile morphological characteristics include small and rounded leaves that become increasingly larger and more spatulated in the adult stage (Bowman, 1993). As a plant progresses through its life cycle, these developmental stages are experienced in a staggered manner along the body of the plants, leading to juvenile and adult stage morphologies existing along the same plant, a phenomenon described as heteroblasty (Huijser and Schmid, 2011). Once adult vegetative growth is concluded, the plant proceeds to the next phase: the reproductive phase, also known as floral transition.

The reproductive organ of *Arabidopsis*

Floral transition is characterized by the transition from vegetative to reproductive development. The SAM develops into an inflorescence meristem, which will laterally differentiate into floral meristems, each with a whorled organ primordium (Araki, 2001). The *Arabidopsis* flower is comprised of four whorls of organs, of which includes the outermost sepals, petals, stamens, and the innermost two fused carpels (Ferrándiz et al., 1999) (Figure 2A). The fused carpels make up the gynoecium, the female reproductive organ of the flower, that can be fertilized to eventually develop into the fruit (Dinneny and Yanofsky, 2005).

Tissue organization in the *Arabidopsis* fruit facilitates gynoecium fertilization, developed seed protection and nourishment, and eventually seed dispersal via dehiscence. The gynoecium is subdivided into three regions; the stigma at the top, the style, then the ovary (Figure 2B). The ovary encloses the developing ovules, and on its outer layer three tissue types can be distinguished: the meristematic replum in the center, the valves at the side, and the valve margin demarcating the former tissue types. Within the replum lies the septum, which separates the ovary into two chambers called locules, along which are the ovules. The valve margin is composed of the adaxial separation layer and the abaxial lignified layer. At full length and maturity, these valve margin tissues undergo mechanical and enzymatic processes that causes the dehiscence of

the valves from the replum, releasing the developed seeds (Roeder and Yanofsky, 2006).

Current genetic network regulating *Arabidopsis* fruit morphogenesis

There exists a large battery of regulatory genes that are required for the correct formation of the gynoecium and fruit (Figure 3). They belong to different transcription factor families and are regulated interdependently. Each gene set is responsible for orchestrating the formation of each tissue type within the gynoecium. The corresponding expression pattern of these genes and their inhibitory interactions allow for correct tissue differentiation (Dinneny and Yanofsky, 2005). The Yanofsky lab has made much headway into the understanding of this regulatory network (Dinneny et al., 2005). However, and taking into consideration the sophistication of fruit morphogenesis is likely that many genes still remain undiscovered.

The valve margin is specified by the combined functions of *SHATTERPROOF1*, *SHATTERPROOF2* (*SHP1*, *2*), *ALCATRAZ* (*ALC*), and *INDEHISCENT* (*IND*) (Liljegren, 2000; Rajani and Sundaresan, 2001; Liljegren et al., 2004). *SHP1* and *SHP2* are MADS-box transcription factors responsible for cellular differentiation into the lignified and separation layers of the valve margin. They are genetically redundant for each other, and a *shp1, 2* double mutant produces an improper valve margin causing the fruit to be unable to dehisce (Liljegren,

2000). *IND* codes for a bHLH transcription factor that is heavily involved in the differentiation of both the separation and lignified layers; an *ind* mutant produces indehiscence as well, albeit the lack of valve margin tissue identity is exacerbated compared to the *shp1, 2* double mutant (Liljegren et al., 2004). *ALC* is another bHLH transcription factor but is only responsible for the differentiation of the separation layer. As such, an *alc* mutant produces a weaker indehiscent phenotype than the *ind* and *shp1, 2* mutants (Rajani and Sundaresan, 2001). *SHP1* and *SHP2* are found to be upstream positive regulators of both *IND* and *ALC* (Robles and Pelaz, 2005). Because of their roles in the differentiation of the separation and lignified layers making up the valve margin, these four genes are heavily tied to the process of fruit dehiscence in *Arabidopsis*.

Valve differentiation is mediated by the activity of *FRUITFULL* (*FUL*) (Gu et al., 1998). *FUL* is a MADS-box transcription factor that plays an important regulatory role in the valve as well as floral meristem identity during fruit morphogenesis (Ferrándiz, 2000a). In the valve, *FUL* downregulates the expression of the above-mentioned valve margin identity genes (Ferrándiz, 2000b). In *ful* mutants, valve differentiation fails and valve cell do not elongate whereas replum normally develops, causing the fruit to develop with a zigzag conformation (Ferrándiz, 2000a). Malformed valves lead to shortened fruits incapable of holding in its normal amount of seeds, ripping apart prematurely (Gu et al., 1998). Meanwhile, transgenic plants that over-express *FUL* produces an ectopic valve cell development. In the quintuple *ful shp1, 2 in alc* mutant, the *ful* mutant

phenotype is almost restored to wildtype morphology (Liljegren et al., 2004). Because of its role in repressing the valve margin identity genes, *FUL* is necessary for the development of valve tissue (Dinneny et al., 2005).

In the replum, *REPLUMLESS* (*RPL*) downregulates the valve margin identity genes (Robles and Pelaz, 2005; Roeder and Yanofsky, 2006). *RPL* is a BELL1 homeodomain transcription factor that has an analogous role in the replum as *FUL* does in the valve. A *rpl* mutant has valve margin cells in the replum, and a *ful rpl* double mutant has valve margin cells in both valve and replum regions. This further supports that the demarcation of the valve margin is regulated by *FUL* repressing the valve margin identity while *RPL* does so in the replum. In conjunction with *RPL*, the KNOX-family gene *BREVIPEDICELLUS* (*BP*) also works to promote replum formation (Alonso-Cantabrana et al., 2007). When *BP* is over-expressed, the repla are enlarged. These two genes *BP* and *RPL* then make up the replum identity genes. Along with the valve margin identity genes, they are down-regulated by the floral homeotic gene *APETALA2* (*AP2*). In an *ap2* mutant, the repla and valve margins were found to be larger than normal (Ripoll et al., 2011), indicating that it down-regulates replum and valve margin identity genes.

Upstream of the regulatory pathway, *BP* is down-regulated by *ASYMMETRIC LEAVES1* (*AS1*) and *ASYMMETRIC LEAVES2* (*AS2*) (Byrne et al., 2000; Byrne et al., 2002; Guo et al., 2008; Ori et al., 2000; Semiarti et al., 2001). *AS1* is an MYB transcription factor while *AS2* is a *LATERAL ORGAN BOUNDRY* (LOB) domain

protein. As such, *as1* or *as2* mutants produce fruits with enlarged repla and diminished valves (Alonso-Cantabrana et al., 2007). Additionally, KNOX-family genes *KNAT2* and *KNAT6* are also down-regulated by the replum identity genes in the replum (Ragni et al., 2008). The C2H2 Zinc-finger transcription factor *JAGGED* (*JAG*) and YABBY genes *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3* (*YAB3*) cooperate redundantly with *AS1, 2* in controlling fruit development (Dinneny et al., 2005). Their decreasing levels of expression across the valve, valve margin, and replum allow for differential regulation of the respective identity genes, dubbed the *JAG/FIL* gradient. Their high and medium levels in the valve and in the valve margin upregulates *FUL* and the valve margin genes while their low levels in the replum allows for increased *BP/RPL* expression. The *fil yab* double mutant produces fruits with small valves and enlarged repla, a familiar phenotype reminiscent to that of the *ful* mutant (Dinneny et al., 2004). To add another layer to this gradient control, the WUSCHEL-related homeobox 13 (*WOX13*) gene's expression decreases across the valve, valve margin, and replum, down-regulating *JAG/FIL* levels (Romera-Branchat et al., 2012). When *WOX13* is over-expressed, the fruit's phenotype resembles that of *FIL, YAB3,* and *JAG* knockout mutants, with ectopically developed repla in the valve regions while in *wox13* mutants, the valve and valve margin tissues develop more medially with shrunken repla. It has also been demonstrated by a previous Master's student of our lab that the *JAG/FIL* activity is down-regulated by the Auxin Response Factors 10, 16,

and 17 (*ARF10, 16, 17*), and that these genes are also post-transcriptionally repressed by microRNA 160 (Bailey, 2012).

A role of small RNAs in fruit morphogenesis

While a lot of non-coding RNAs are thought be "molecular junk", many have been demonstrated to have important regulatory roles in gene expression. MicroRNAs (miRNAs) are 20-24 nucleotides long and they are able to post-transcriptionally repress genetic activity (Bartel, 2004). miRNAs are transcribed by RNA Polymerase II in the nucleus, forming the hairpin-structured primary transcript pri-miRNA, which is then processed into a small, double-stranded RNA segment to be exported out to the cytoplasm (Chen, 2005). One of the strands is degraded, while the other, the guide strand, complexes with other proteins to form the mature miRNA. The mature ribonucleoprotein complex is now able to recognize its target mRNA sequence and carry out one of two functions: degradation of the target transcript or blocking its transcription.

The Yanofsky lab has characterized some miRNAs and their roles in fruit morphogenesis. As mentioned above, miRNA 160 (miR160) plays an important role in regulating fruit tissue differentiation in *Arabidopsis*. miR160 post-transcriptionally represses the expression of *ARF10, ARF16,* and *ARF17*, and these transcription factors have been shown to down-regulate the *JAG/FIL* gradient necessary for distinctive tissue differentiation in the fruit (Bailey, 2012; Mallory et

al., 2005; Rhoades et al., 2002; Wang et al., 2005). miR172 is another miRNA that is a part of this regulatory network, contributing to *FUL*'s expression in valve tissue (Ripoll et al., 2015). *FUL*, together with *ARF6* and *ARF8*, upregulates the expression of miR172, which in turn represses the *AP2*'s down-regulating effect on *FUL*, allowing for the valve to develop.

The small signaling peptide CLE

CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR) – RELATED (*CLE*) is a family of small, secreted, intercellular signaling peptides that all share a highly conserved 14-amino acid CLE domain (Cock and McCormick, 2001). In *Arabidopsis, CLE* genes have been shown to be involved in root and shoot apical meristem differentiation and maintenance as well as vascular development (Casamitjana-Martinez et al., 2003; Fiers et al., 2004; Fiers et al., 2005; Fiers et al., 2007; Hobe et al., 2003; Ito et al., 2006; Strabala et al., 2006). Although little is known about *CLE*'s functions in fruit development, expression analysis based on transgenic reporter lines revealed a number of CLE genes expressed in different regions of the fruit (Jun et al., 2010). Similar to miRNAs, CLE peptides are encoded in small loci, which represents a challenge for generating knock-out mutants and do functional studies.

The use of CRISPR/Cas9 in gene editing

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein (Cas) was originally a bacterial immune system against viruses that has since been adapted for specific genome editing (Barrangou et al., 2007; Garneau et al., 2010; Jinek et al., 2012; Mali et al., 2013; Mojica et al., 2000). The mechanism behind this precision editing technique involves a sequence-specific DNA recognition and binding domain combined with an effector domain that cleaves the target side, generating double-stranded breaks (Gasiunas et al., 2012). Double-stranded breaks stimulate DNA repair pathways like non-homologous end joining or homology-directed repair, allowing for gene knockout via random insertion/deletions or gene alteration via sequence replacement respectively (Rouet et al., 1994a; Rouet et al., 1994b) (Figure 4). Cas9 is an endonuclease able to recognize a specific sequence due to its guiding-RNA (gRNA) binding to the complementary target sequence, in addition to the Cas9's direct interactions with a short protospacer-adjacent motif (PAM) adjacent to the target site (Bolotin et al., 2005). Once bound, the Cas9 will cleave, generating a double-stranded break in the target site approximately three nucleotides upstream from the PAM site. While the Cas9 can only cleave target sequences that contain the PAM site, the motif occurs once every eight nucleotides in the genome (Hsu et al., 2014).

MATERIALS AND METHODS

Plant materials

All *Arabidopsis thaliana* lines used and generated had Columbia (Col-0) as the wild type. Plant materials included in this study were *C9αmiR172D* (this work), *C9αmiR160A* (this work), the driver line *FUL::LhG4* was made in our lab, *OP::C9αmiR160A* (this work), *CLE25::GUS* (Jun et al., 2010), *C9αCLE25*, ful-2 (Ferrándiz et al., 2000a), ful-6 (Ferrándiz et al., 2000a), and cle25cr1 was sent to us by Yamaguchi et al., 2017.

Cloning strategies

Gene-specific gRNA sequences for *C9αmiR172D, C9αmiR160A*, and *C9αCLE25* were designed with the tools from CRISPR-PLANT (https://www.genome.arizona.edu/crispr/CRISPRsearch.html)(Xie et al., 2014) (Table 1). Dual gRNA sequences for *miR172D* (AT3G55512) and *miR160A* (AT2G39175) were amplified with the above-mentioned Phusion Taq Polymerase using Col-0 genomic DNA as template. gRNA sequences were then cloned into the T-DNA delivery vector pJJJ2 using our own DNA Assembly protocol. The *Agrobacterium* T-DNA delivery vector pJJJ2 for the dual gRNAs and Cas9 (vector design in Figure 9) was designed by the collaboration between our lab's Dr. Juan José Ripoll-Samper and Dr. José Pruneda-Paz of the Pruneda-Paz lab. For generating *OP:: C9αmiR160A*, the gRNAs for *C9αmiR160A* was cloned into the

pJJJ3 vector, a version of the pJJJ2 vector that uses the *OP* promoter instead of *UBQ*.

Generating and isolating transgenic plants

Each resulting pJJJ2 (or pJJJ3 in the case of *OP:: C9αmiR160A*) T-DNA construct was co-transformed into *Agrobacterium tumefaciens* (AGL-0) with the helper plasmid pSOUP (Hellens et al., 2000) via electroporation. Col-0 were used as background *Arabidopsis thaliana* plants for the floral dipping method (Clough and Bent, 1998). T1 transgenic plants for *C9αmiR172D, C9αmiR160A, OP:: C9αmiR160A*, *C9αCLE25* were sown on MS plates containing 20mg/mL Hygromycin, where viable seedlings were then transplanted onto soil after a 1-2 weeks. *FUL>>OP::C9αmiR160A* plants were double-selected for first with 20mg/mL Hygromycin plates, then subsequently sprayed with BASTA (120mg/mL ammonium sulfate; Finale, AgrEvo, Montvale, NJ) twice to three times a week for 2 weeks.

GUS staining

Fruit tissues were treated as previously described (Ripoll et al, 2011). Whole-mount microscopy for taking pictures of stained tissues used a standard dissecting scope with adapted camera module.

RESULTS

I. The role of miRNA in *Arabidopsis* fruit development

As previously mentioned, microRNAs (miRNAs) have been shown to have regulatory functions in *Arabidopsis* fruit development. Their important roles in indirectly promoting valve tissue development through post-transcriptional repression have been characterized. However, due to the small size of the miRNA gene loci, it has proven difficult to generate knock-out mutants for them, with past experiments indirectly repressing their activities by generating miRNA-resistant mutant target genes or by sequestering miRNA transcripts. Now that the CRISPR/Cas bacterial immune system has been adapted for gene editing, we will be able to directly target the miRNA gene.

Members of the Yanofsky lab have shown that *miRNA172 (miR172)* ~~indirectly~~ promotes valve development by repressing *AP2*'s inhibitory effect on *FUL*, the valve identity gene (Ripoll et al., 2015). The *miR172* family is comprised of five variants. *miR172C* was the variant shown to have a role in up-regulating valve identity gene activity.

In the same study, *miR172D* activity was detected in the ovules and seeds. This expression pattern is consistent with the phenotype observed in a mild *mir172* mutant background described in a previous study. In order to further gain insight into the role of *miR172D* in fruit, we decided to follow a functional strategy by using genome editing tools.

I. 1. Optimizing CRISPR/Cas9 to reaffirm the role of miR172D in fruit development

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein (Cas) is the bacterial antiviral immune system. It has been adapted for gene editing, vastly improving target precision and ease with which to induce mutations (Barrangou et al., 2007; Garneau et al., 2010; Jinek et al., 2012; Mali et al., 2013; Mojica et al., 2000). The Cas9-gRNA complex can be programmed to specifically target any target sequences with the commonly occurring protospacer-adjacent motif (PAM), generating a double-stranded break that induces an insertion/deletion mutation.

Using CRISPR/Cas9 gene editing technology, we wanted to target the *miR172D* locus with the hope to generate a knock-out mutant. Originally, when CRISPR/Cas9 was described in its use for targeting a specific genomic region, only one guide RNA (gRNA) was described to be used (Jinek et al., 2012). In our case, we employed two gRNAs to target the *miR172D* locus, aiming to generate a deletion within the *mir172D* coding region (Figure 5A), which will hopefully ensure a knock-out of *miR172D* function. Based on previous observation, we expect the *C9amiR172D* resulting mutant to further enhance the phenotype seen in the mild *miR172D* mutant allele. The Materials and Method section contains details as to how we delivered the Cas9 construct and generated the *C9amiR172D* mutant.

I. 2. *C9amiR172D* mutant phenotype consistent with the pattern of *miR172D* mutant phenotype

Upon examining the developed fruits of *C9amiR172D* mutants, we found that the silique was noticeably shorter (Figure 5B). However, unlike in mutants with down-regulated valve identity genes where the valves were tighter due to the same number of seeds being compressed inside diminished valves, the smaller valves in our *C9amiR172D* mutant did not appear tightly bound. By dissecting the valves, we observed that there were little to no seeds, and in their place were many undeveloped ovules (Figure 5C). This phenotype was consistent with previous observations and the expression pattern of the *GUS* reporter for *mir172D* (Ripoll et al., 2015).

We wanted to examine what kind of mutation was made by the Cas9, so we sequenced the *C9amiR172D* target region. Based on our dual-gRNA design, we expected to see a ~~large~~ deletion in the locus. However, we observed only a single nucleotide substitution at the target region of one of the gRNAs (Figure 5D). While this was not as robust a mutation as we desired, it was still sufficient to produce a strong phenotype.

II. Further implementation of CRISPR/Cas9 in knocking out *miRNA* function

Our moderate success with using CRISPR/Cas9 to knock out *miR172D* function encouraged us to continue implementing this system. Based on previous

findings and the work of one of our past Master's student. *miRNA160 (miR160)* has been shown to be involved in the fruit development regulatory network (Bailey, 2012; Mallory et al., 2005; Rhoades et al., 2002; Wang et al., 2005). The *miR160* family contains three variants: A, B, and C. *miR160A* is known to repress Auxin Response Factors (*ARF*) 10, 16, and 17, which have been shown to inhibit the *JAG/FIL* gradient that upregulates the valve identity gene FUL. In this way, *miR160A* is indirectly promoting *Arabidopsis* valve development. As such, this makes *miR160A* a target of interest for our study. As with *miR172D*, we will also be implementing the dual-gRNA CRISPR/Cas9 system in targeting the *miR160A* locus (Figure 6A).

II. 1. *C9α**miR160A* mutant phenocopies miRNA-resistant *ARF* mutants in fruit morphology

In past experiments, miRNA-resistant mutants of *ARF10* and *ARF16* were generated (Liu et al., 2007; Wang et al., 2005). The resulting transgenic plants developed smaller overall fruit size, with the characteristic smaller and tighter valves that mutant fruits have when the valve identity gene regulatory network is down-regulated (Figure 6B). Because of the relationship between these *ARFs* and *miR160A*, we predict that our knocking out *miR160A* will also result in similar phenotypes, not unlike those of *jag fil* double mutants (Dinneny et al., 2004).

Consistent with those expectations, *C9αmiR160A* phenocopied the miRNA-resistant *ARF*s (Figure 6C). We proceeded to sequencing the *miR160A* locus of our knock-out mutants and found that the Cas9 has generated a very robust deletion (Figure 6D). Along the way, we also observed another mutant phenotype: the reduction in size of the seedling (Figure 6E). This observation challenged us to further optimize our CRISPR/Cas9 toolkit so that Cas9 mutations can be restricted to a specific tissue type.

II. 2. Employing the pOP/LhG4 trans-activation system in designing a tissue-specific CRISPR/Cas9 gene editing

The two-component lac- and Gal4-based (pOP/LhG4) trans-activation system has been used in the past for tissue-specific expression of any gene (Moore et al., 1998). Since then, this system has been adapted and optimized for use in *Arabidopsis* as well, including in the works of past members of our lab (Bailey, 2012; Baroux et al., 2005). By using a tissue-specific "driver" promoter to express *LhG4*, the *OP* promoter attached to the "operator" gene of interest can be activated, inducing expression the expression of that gene. For our experiment, the use of the promoter component of valve identity gene *FUL* as the driver with the Cas9 construct as the operator should allow us to restrict Cas9-mediated knock-out mutations to the valve tissue. The Yanofsky lab has coined the name "Tissue-Specific Inducible Knock-Out" (TSIKO) for this system (Ripoll et al., unpublished).

In order to knock out *miR160A* only in the valve tissue, we crossed the driver line *FUL::LhG4* with the operator line *OP::C9amiR160A* (Figure 7A). These mutant lines were generated according to our protocol in the Materials and Methods section. We then carefully extracted only valve tissue and root tissue so that we can sequence their *miR160A* target regions. Since *FUL* has not ever been shown to be expressed in the roots, we expect to only see mutations in the valves, with no changes in the roots *miR160A* target region. Unfortunately, phenotypic data of the *FUL>>::C9amiR160A* is available. However, sequencing data revealed mutations in the *miR160A* locus of root tissue, something we did not anticipate (Figure 7B).

III. Expanding the fruit development regulatory network using CRISPR/Cas9

Our fruitful endeavors with CRISPR/Cas9 gene editing for reaffirming the roles of miRNA in fruit development emboldened us to seek out more small regulatory genes to examine. This search pointed us towards the *CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR) – RELATED (CLE)* family of small inter-cellular signaling peptides. The *CLE*s have characterized roles in plant stem cell differentiation, and has been associated with other *Arabidopsis* plant functions (Casamitjana-Martinez et al., 2003; Fiers et al., 2004; Fiers et al., 2005; Fiers et al., 2007; Hobe et al., 2003; Ito et al., 2006; Strabala et al., 2006). However, despite comprehensive expression analysis demonstrating various *CLE* activities in

the fruit, little functional data is available for their roles in fruit development (Jun et al., 2010). As such, we would like to implement our adapted CRISPR/Cas9 gene editing toolkit to explore their potential functions.

III. 1. Acquiring a knock-out mutant for *CLE25* with CRISPR/Cas9

Our *GUS*-reporter line showed *CLE25* activity in the valve margins (Figure 8A). The valve margins are of particular importance in the dehiscence of fully developed fruits, and its proper genetic regulation is tightly bound to the development of the nearby valve and replum tissues. Thusly, we seek to explore its potential role in valve margin development by knocking it out with our dual-gRNA CRISPR/Cas9 strategy. Around this time, we received more *CLE* mutant alleles from another group of researchers who have recently generated a *CLE* mutant library using CRISPR/Cas9 (Yamaguchi et al., 2017)). Similarly, they also employed two gRNAs in targeting the *CLE25* locus with a slight difference in target regions selection (Yamaguchi et al., 2017) (Figure 8B). With this, they were able to introduce a single nucleotide deletion into the *CLE25* gene. Although not shown here, both our and their *CLE25* mutant (*cle25cr1*) displayed no phenotypes differing from the wild type Col-0. We ultimately proceeded with our coming experiments using *cle25cr1*.

III. 2. Mutations in *CLE25* partially rescued *ful* fruit phenotype

Valve margin genes are known to be down-regulated by valve and replum identity genes in their respective tissues. If *CLE25* is a part of the valve margin identity genes, then knocking out its expression alone is likely not sufficient to induce a phenotype; this idea is consistent with our observation of the *CLE25* mutant fruit. *FUL* mutant fruits have been shown to have ectopically expressed valve margin tissues in the valves (Liljegren et al., 2004). Based on this context, we crossed *cle25cr1* to *ful* mutant. If *CLE25* is part of the valve margin identity genes, we should observe a reduction in the *ful* phenotype in the double mutant.

While the *ful-2 cle25cr1* fruits were slightly larger than *ful-2* fruits on average, the double mutant still retains much of the typical *ful* valve features, an indication of ectopically developed valve margins (Figure 8C). This is likely due to the high redundancy of *CLE* genes, prompting us to cross *cle25cr1* with a milder *FUL* mutant, ful-6. *ful-6 cle25cr1* fruits were not only closer to wild type in size, but their valves were also closer in morphology as well. This is a strong indication that *CLE25* does indeed have a role in valve margin development, similarly to how the loss of activity for the valve margin identity genes *IND, SHP,* and *ALC* suppressed the *ful* fruit phenotype (Liljegren et al., 2004).

The difference in *ful* phenotype rescue between the two double mutants could be explained as *CLE25* collaborating redundantly with another *CLE* gene in regulating valve margin development. For example, *CLE21* has also been

shown to be expressed in the valve margins (Jun et al., 2010). As of right now, we have successfully generated Cas9-induced knock-out mutants for other *CLE* genes of interest but have not been able to experiment with them yet. We are hopeful that further experimentation will lead to detailed identification and characterization of *CLE* functions in this regulatory network.

DISCUSSION

I. Adapting CRISPR/Cas9 gene editing to reaffirm the role of *miR172D* in fruit development

CRISPR/Cas9 is a gene editing tool adapted from bacterial antiviral immune system that allows for precise sequence targeting that generates insertion/deletion mutations through blunt-end double-stranded breaks (Barrangou et al., 2007; Garneau et al., 2010; Jinek et al., 2012; Mali et al., 2013; Mojica et al., 2000). This molecular tool allows us to target small regulatory genes that were previously unable to be knocked out.

Past work in our lab has demonstrated the important role that *miR172* has in dis-inhibiting the valve identity gene *FUL* (Ripoll et al., 2015). In that study, they observed *miR172D* activity in the ovules and seeds. In the *mir172d-1* mutant, they also saw a mild phenotype that resulted in smaller seeds. Based on this data, we generated the *C9αmiR172D* mutant hoping to recapitulate this phenotype and confirm the role that *miR172D* has in ovule and seed development. We optimized the CRISPR/Cas9 gene editing method to use two gRNAs instead, hoping to generate a large deletion in the intermediate region between the gRNAs.

The *C9αmiR172D* mutant fruits were observed to have aborted ovules, resulting in very few seeds being developed (Figure 5B, C). It was also of note that the mutant fruit was shorter, although the valves did not display the characteristic *ful* mutant phenotype, likely due to the lack of seeds that normally would cause

the *ful* valves to tear. In consistency with the observed pattern in *mir172d-1* mutants, as *miR172D* function is increasingly disrupted, ovules and seeds are even less developed. This observation further reinforces the role that the *miR172* family has in *Arabidopsis* fruit development.

Our sequencing data for the target regions of *C9αmiR172D* showed only a single nucleotide substitution instead of the expected large deletion (Figure 5D). However, this small mutation was still sufficient to induce the above phenotype. It has been shown that miRNA activity is sensitive to nucleotide changes in the pri-miRNA ring-loop structure, which we suspect is also the case here (Liu et al., 2008). As for the less-than-expected mutation, it shows that we should optimize our gRNA selection as Cas9 efficacy is affected by gRNA architecture (Hsu et al., 2013).

Currently, we also have other *C9αmiR172D* mutant alleles in development. This will allow us to see if a large deletion as expected would produce an even stronger phenotype than seen here.

II. 1. *C9αmiR160A* phenocopies miRNA-resistant *ARF* mutants in fruit morphology

The work of one of our previous Master's students identified the *ARF 10, 16,* and *17* as down-regulators of the *JAG/FIL* gradient (Bailey 2012). Past data showed *miR160A* repressing these *ARF,* bridging the *miR160* family into the fruit regulatory network as the indirect promoter of the *JAG/FIL* gradient (Mallory et al., 2005; Rhoades et al., 2002; Wang et al., 2005). In further developing our

CRISPR/Cas9 toolkit, we selected *miR160A* for a knock-out study, re-implementing our dual-gRNA strategy here as well.

In past experiments demonstrating *ARF10, 16,* and *17* as targets of *miR160A* repression, miRNA-resistant mutants for *ARF10* and *ARF16* were created (*mARF10, mARF16*), which displayed fruit phenotypes similar to those of *jag fil* double mutants (Dinneny et al., 2004; Liu et al., 2007; Wang et al., 2005) (Figure 6B). As such, we expect our *C9a*m*iR160A* mutant to have similar fruit phenotypes as those of *mARF10* and *mARF16*, which was what was observed (Figure 6C). Unlike *C9a*m*iR172D,* the sequencing result of the target locus in *C9a*m*iR160A* showed a deletion consistent with the gRNA design (Figure 6D). Our functional analyses have provided additional evidence to further support a role for miR160 in fruit development.

II. 2. Developing Tissue-Specific Inducible Knock-Out system to further improve our CRISPR/Cas9 toolkit

The *C9a*m*iR160A* mutant plant was also observed to have a dramatic reduction in seedling size (Figure 6E). This additional mutant phenotype and the fact that *C9a*m*iR160A* had a robust deletion mutation prompted us to use *miR160A* as the candidate for developing a Tissue-Specific Inducible Knock-Out (TSIKO) system (Figure 7A). In order to achieve this, we employed the pOP/LhG4 trans-activation system. This two-component system allows for the tissue-specific

activation of a gene, and has since been adapted for use in *Arabidopsis thaliana* among other model organisms (Baroux et al., 2005; Moore et al., 1998).

Using the promoter of the valve identity gene *FUL* as the driver and the *C9αmiR160A* construct as the operator, we hope to be able to restrict Cas9-mediated *miR160A* knock-out to the valve tissue. Although we have no available phenotypic data of the *FUL>>C9αmiR160A* line, our sequencing data detected Cas9 activity in the roots (Figure 7B), an unexpected result due to *FUL* having no past data showing its expression in *Arabidopsis* root tissue.

We came up with two hypotheses to rationalize this phenomenon: that the Cas9/gRNA complex has somehow migrated across tissues, or that *FUL* is also expressed in the root tissue, albeit at a very low level undetectable by past *GUS* expression analysis. We quickly dismissed the former hypothesis due to the fact that the Cas9 is designed with a Nuclear Localization Sequence in it, immediately taking the expressed ribonucleoprotein complex to the nucleus which should not allow it to travel across tissue. Currently, we have already generated new tissue-specific driver lines that utilizes the promoter components of the roots-only *RCH1* and green tissues-only *FRO7* genes (Casamitjana-Martinez et al., 2003; Mukherjee et al., 2006). While we do not have data for these driver lines crossed with the *OP::C9αmiR160A* operator line yet, we are confident that they will improve the TSIKO system and hint at what caused the non-tissue-specificity in our *FUL>>::C9αmiR160A* line.

III. Expanding the *Arabidopsis* fruit regulatory network with CRISPR/Cas9

Our success in knocking out the miRNA genes encouraged to functionally challenge additional loci encoding for small regulatory genes and small signaling peptides. Based on available expression data showing some activity in various fruit tissues, we looked at the *CLAVATA3* (*CLV3*)/*ENDOSPERM SURROUNDING REGION* (*ESR*) – *RELATED* (*CLE*) family (Jun et al., 2010). Our own expression data allowed us to select *CLE25*, whose activity was observed in the valve margins (Figure 8A). During the process of generating our own *CLE25* Cas9 knock-out mutant, we received *cle25cr1* from a group of researchers that has generated a collection of *CLE* mutants with CRISPR/Cas9 (Yamaguchi et al., 2017). Both *CLE25* mutant alleles displayed no discernible differences in phenotype from the wild type (data not shown), indicating that there is likely redundant *CLE* activity in the valve margins.

Valve margin genes are tightly regulated by valve and replum identity genes in their respective tissues. Thusly, we crossed *cle25cr1* with the strong *FUL* mutant *ful-2* and the milder *ful*-6. We found that *CLE25* knock-out in a *FUL* mutant background was able to partially rescue the *ful* phenotype (Figure 8C). The difference in phenotype rescue between the *ful-2 cle25cr1* and *ful-6 cle25cr1* double mutants indicates that there are likely other *CLE* genes working redundantly with *CLE25*. Notably, this suppressed *ful* phenotype is very similar to the loss of downstream valve margin gene (*IND* and *ALC*) activities in a *FUL*

mutant background, suggesting that *CLE25* likely belongs downstream of the valve margin regulatory node (Liljegren et al., 2004).

Currently, we have developed more Cas9-mediated deletion mutants for other *CLE*s of interest, including *CLE16* and *CLE21* which have been shown to be expressed in the valves and valve margins respectively (Jun et al., 2010). In the near future, we plan to further characterize the role of *CLE* genes in fruit patterning by

IV. Future prospects

The data from this project has allowed us to expand our molecular toolkit to include the use of CRISPR/Cas9 in an effective manner. Our promising data using the TSIKO system has encouraged us to further optimize this technology and design additional driver lines to more specifically control expression in fruit and thus achieve fruit tissue-specific Cas9 knock-out.

We have also generated Cas9-induced knock-out mutants for more *CLE*s of interest, although we do not have any experimental data for them. Loss-of-function studies in more mutant backgrounds, generating more high order mutant background combining different *CLE* mutants will allow us to more comprehensively characterize the roles of *CLE* genes in fruit.

There are still much to be done, but we are optimistic at the prospect of recruiting the *CLE* genes into the *Arabidopsis* fruit development regulatory network.

APPENDIX A: FIGURES AND FIGURE LEGENDS

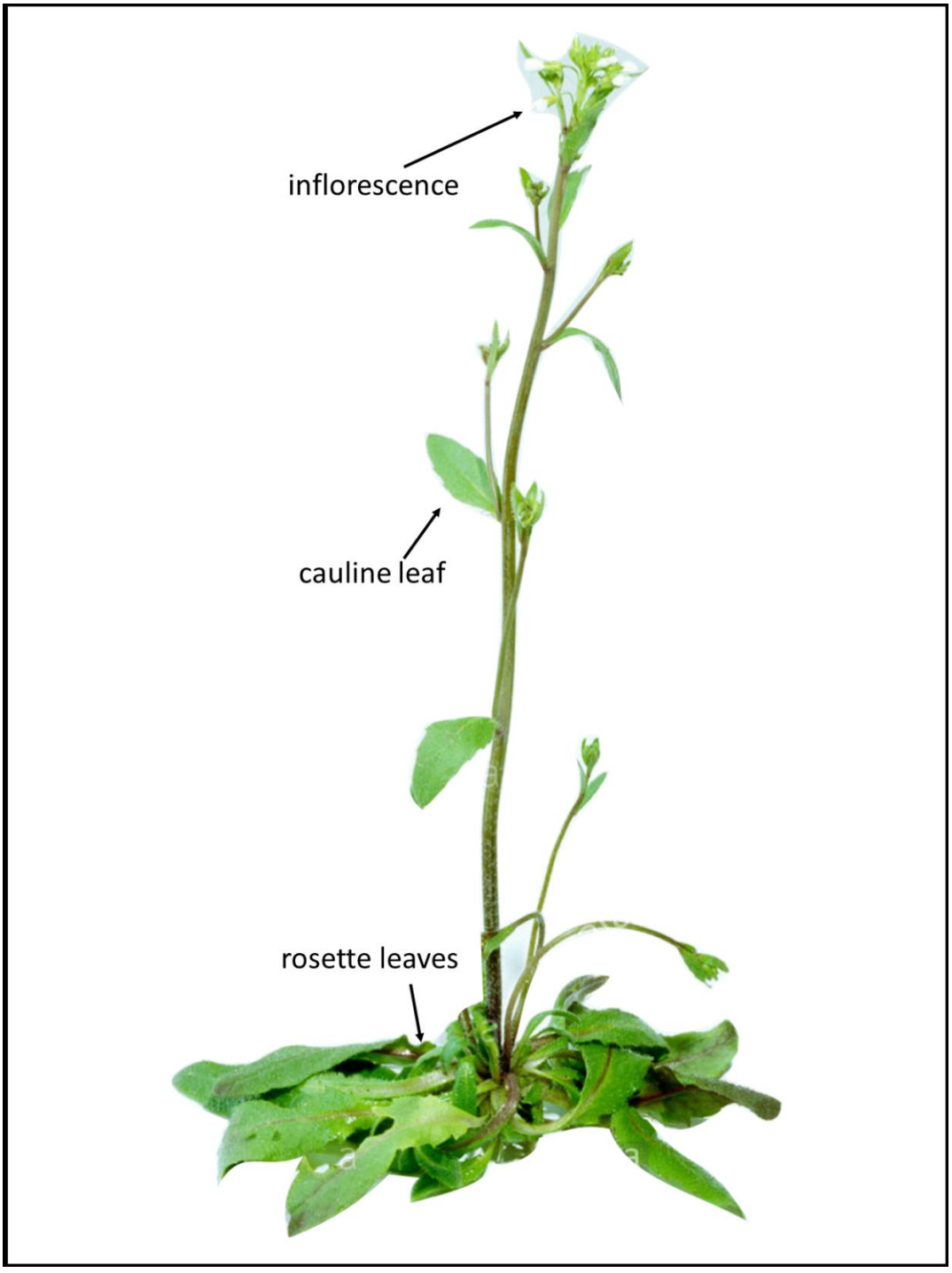


Figure 1. Vegetative Anatomy of *Arabidopsis thaliana*

An adult *Arabidopsis* plant. Each shoot has an inflorescence producing several flowers.

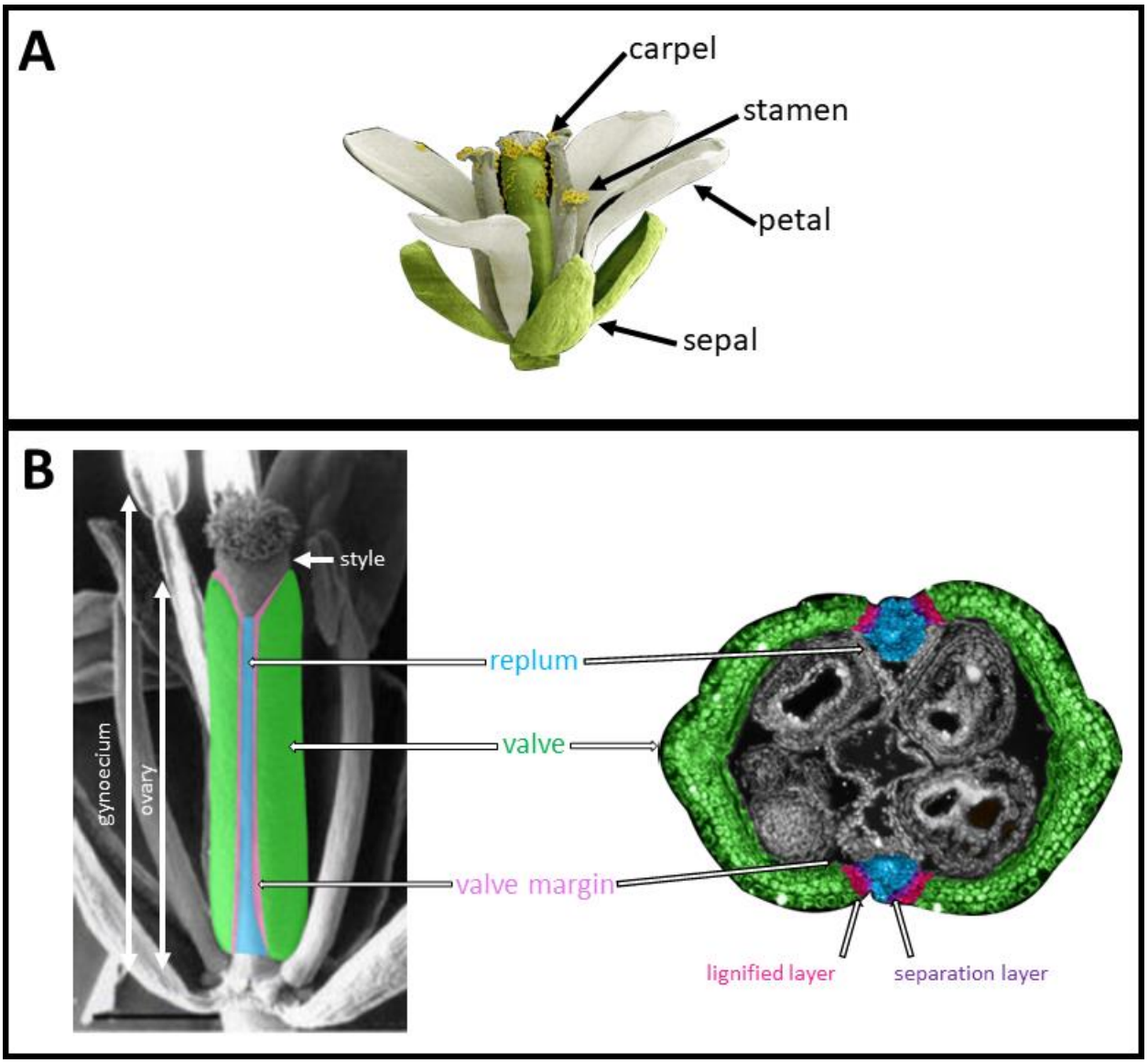


Figure 2. Reproductive anatomy of *Arabidopsis thaliana*

(A) The flower consists of four concentric rings of organs surrounding the central axis. The reproductive organs are in the center, while the protective organs are the outermost layers. **(B)** On the left is a stage 15 fruit, with the main regions of the gynoecium indicated. On the right is a cross-section of a stage 16 fruit, with major tissue regions highlighted and corresponded with where they would be from the outside. Replum is in blue, valve is in green, and valve margin is in pink. The valve margin is composed of two layers: the adaxial separation layer (purple) and the abaxial lignified layer (dark pink).

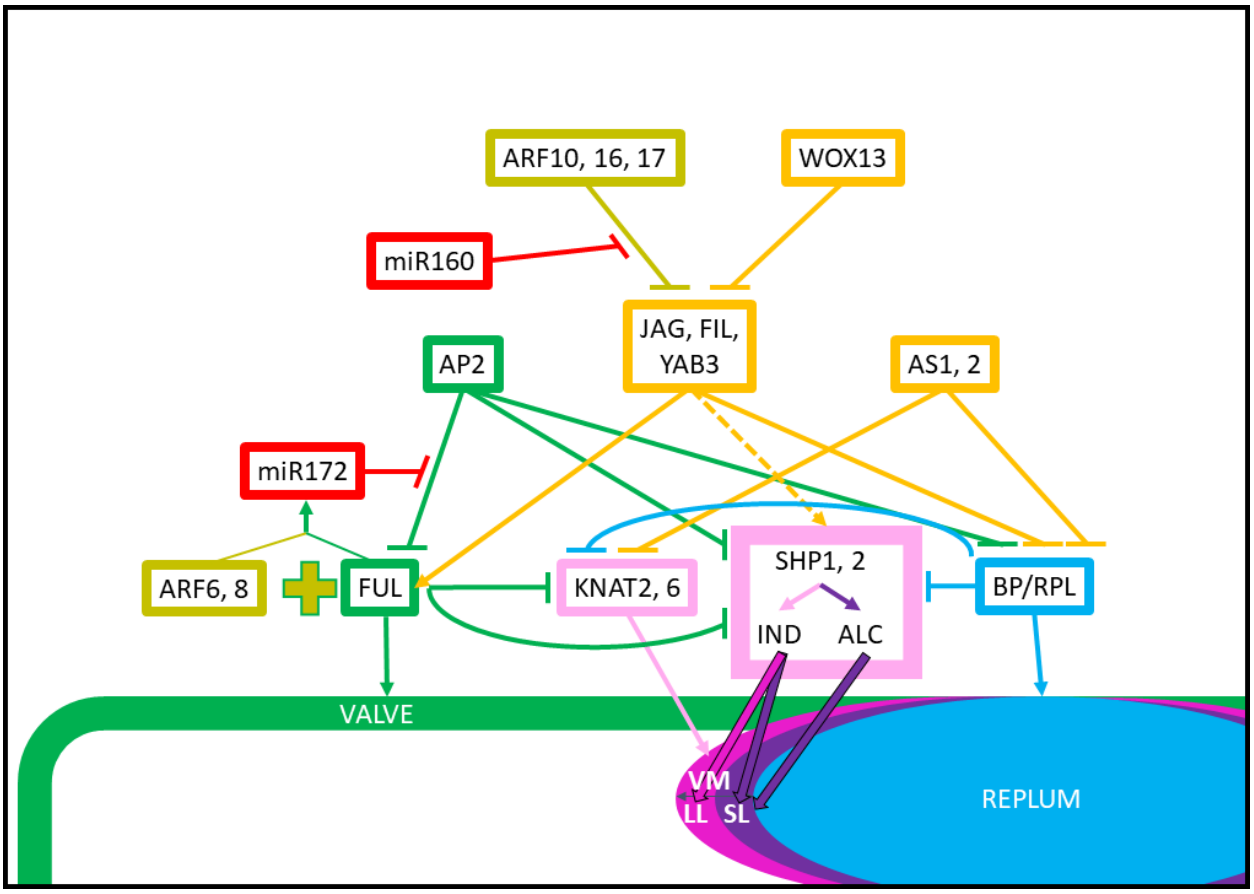


Figure 3. Current genetic network regulating fruit development

The framework model shows the described genes and their regulatory roles in *Arabidopsis* ovary patterning.

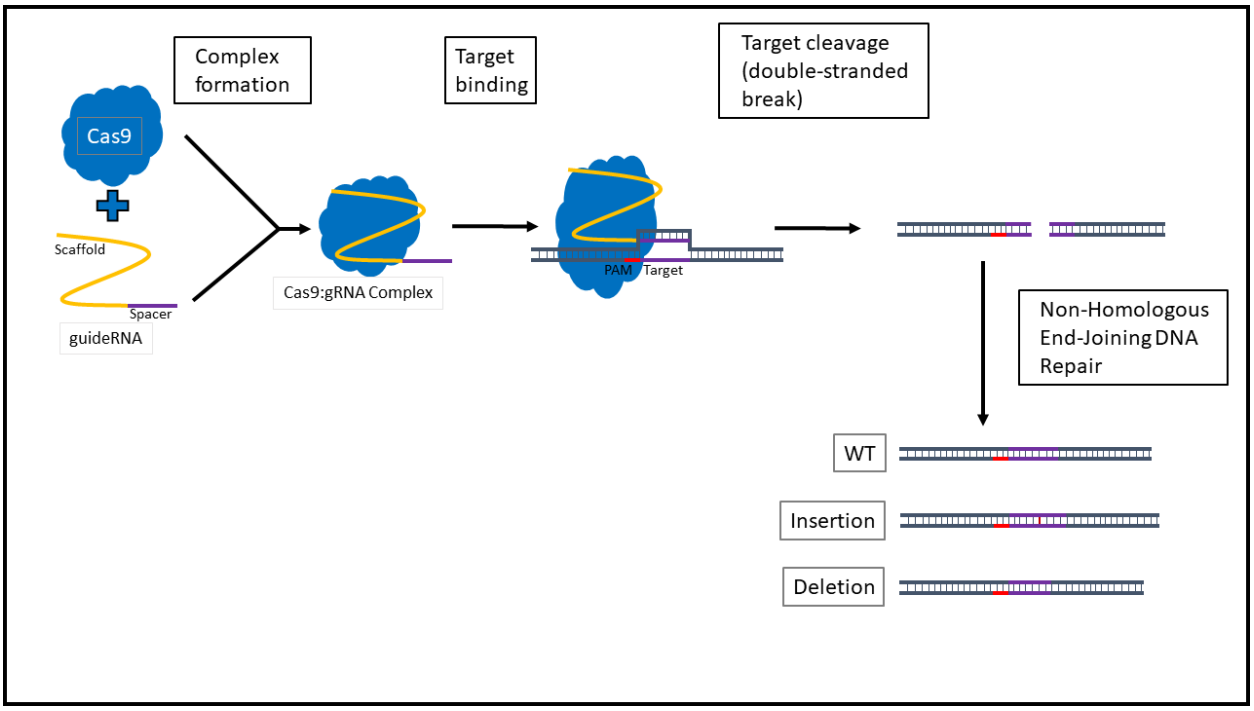


Figure 4. Basic mechanism of CRISPR/Cas9 gene editing

The model shows the main steps of Cas9-mediated gene editing resulting in insertion/deletion mutations.

Figure 5. Cas9-mediated knock-out of *miR172D* resulted in ovule abortion phenotype.

(A) A model showing gRNA targeting strategy in the *miR172D* locus. **(B-C)** Whole mount microscopic images of wild type Col-0 and mutant *C9a**miR172D* fruits. **(D)** Comparison of sequencing results for the target region in the *miR172D* locus between wild type Col-0 plant and *C9a**miR172D* plant.

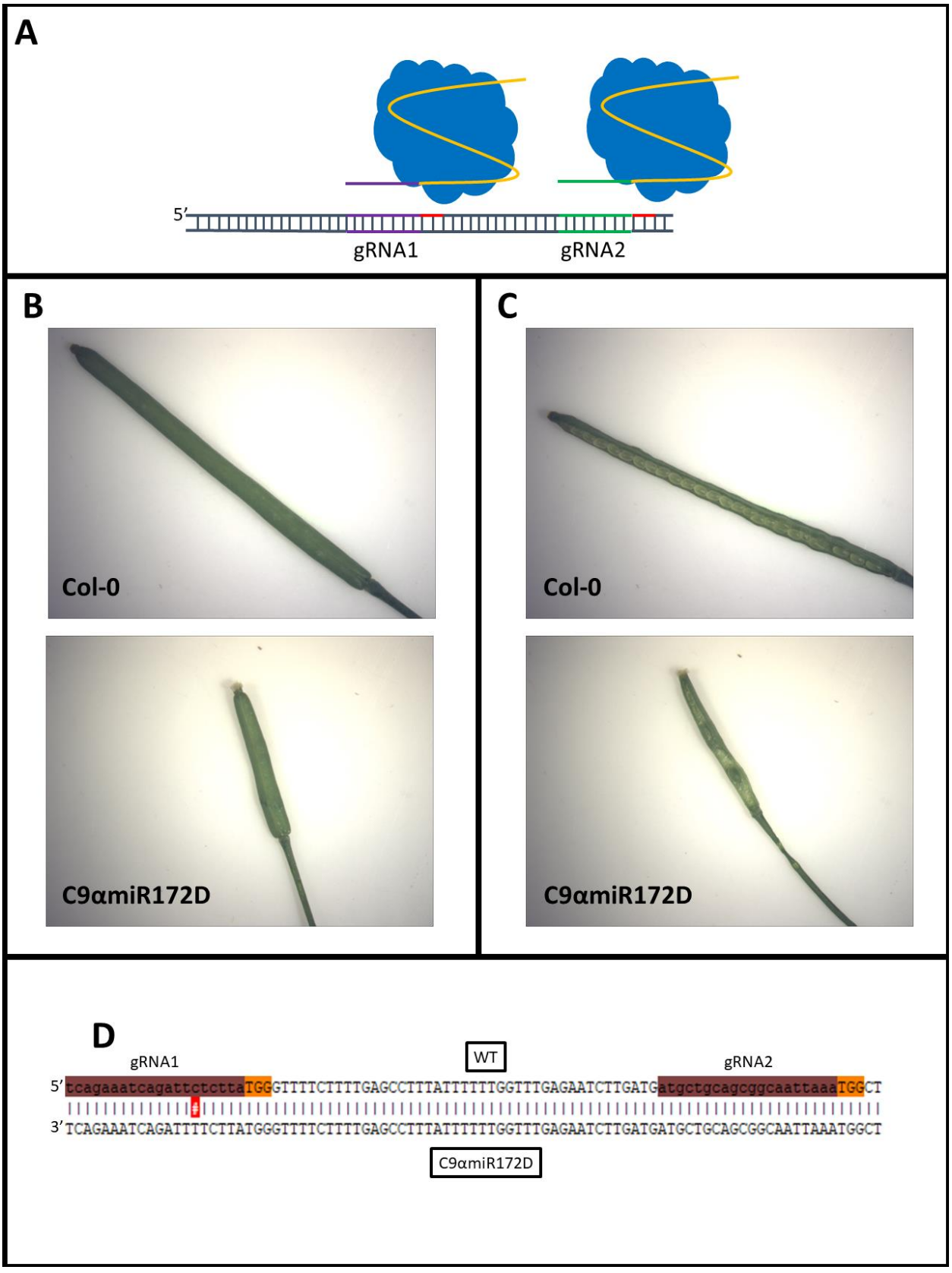
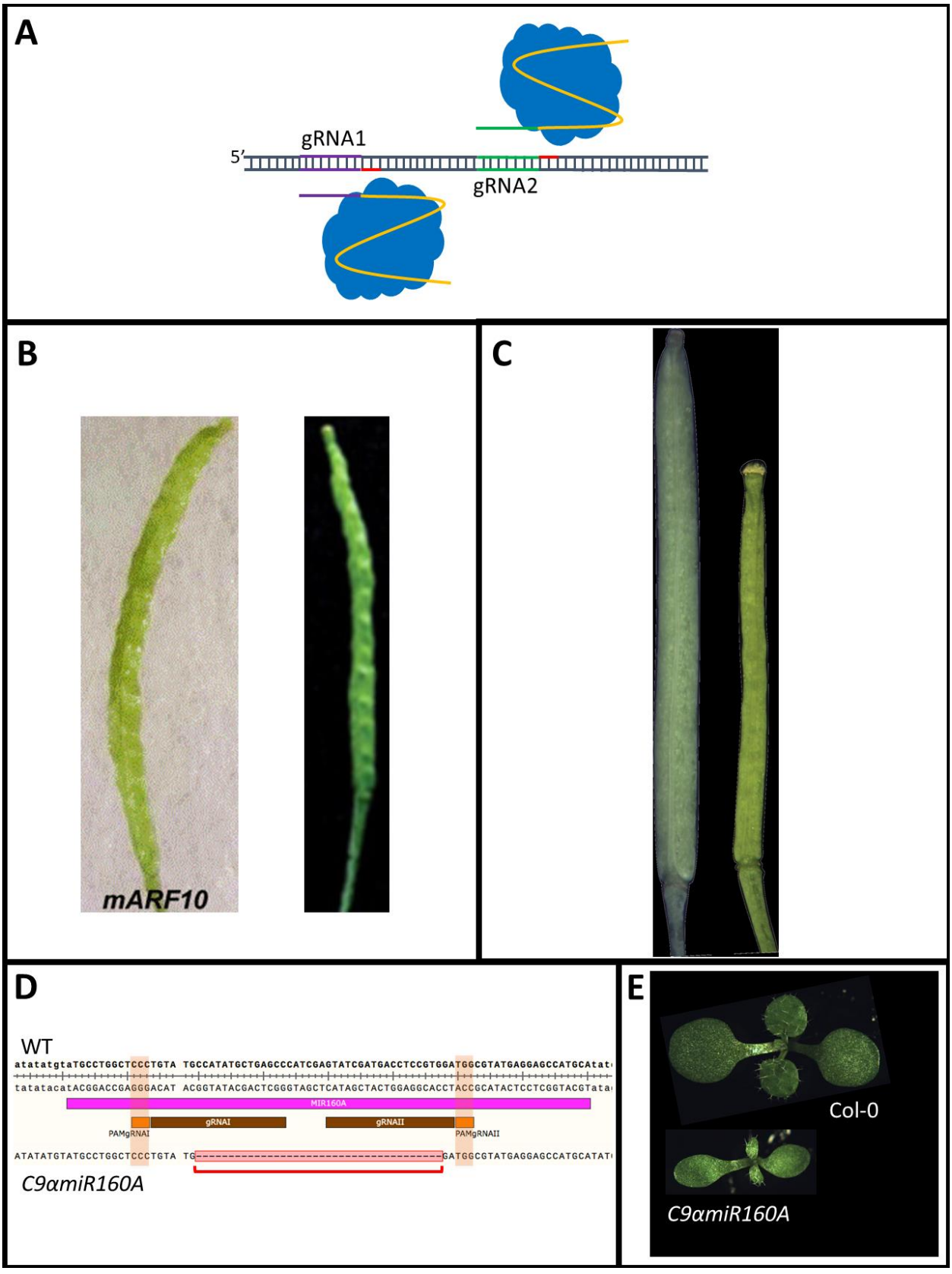

A
5′
gRNA1
gRNA2
B
Col-0
C9αmiR172D
C
Col-0
C9αmiR172D
D
gRNA1
WT
gRNA2
5′tcagaaatcagattctcttaTGGGTTTTCTTTTGAGCCTTTATTTTTTGGTTTGAGAATCTTGATGatgctgcagcggcaattaaaTGGCT
3′TCAGAAATCAGATTTTCTTATGGGTTTTCTTTTGAGCCTTTATTTTTTGGTTTGAGAATCTTGATGATGCTGCAGCGGCAATTAAATGGCT
C9αmiR172D

Figure 6. *C9amiR160A* phenocopies miRNA-resistant *ARF* mutant fruits.

(A) A model showing gRNA targeting strategy in the *miR160A* locus. **(B)** Images of miRNA-resistant *ARF10* and *ARF16* (*mARF10, 16*) fruits from Liu et al., 2007 and Wang et al., 2005 respectively. **(C)** Whole mount microscopic images of wild type Col-0 fruit (left) and *C9amiR160A* fruit (right). **(D)** Comparison of sequencing results for the target region in the *miR160A* locus between wild type Col-0 plant and *C9amiR160A* plant. **(E)** Comparison of wild type Col-0 seedling and *C9amiR160A* seedling at about 16 days.


A
gRNA1
5’
gRNA2
B
mARF10
C
D
WT
atatatgtaTGCCTGGCTCCCTGTA TGCCATATGCTGAGCCCATCGAGTATCGATGACCTCCGTGGATGGCGTATGAGGAGCCATGCAtat
tatatacatACGGACCGAGGGACAT ACGGTATACGACTCGGGTAGCTCATAGCTACTGGAGGCACCTACCGCATACTCCTCGGTACGTata
MIR160A
gRNAI
gRNAII
PAMgRNAI
PAMgRNAII
ATATATGTATGCCTGGCTCCCTGTA TG
GATGGCGTATGAGGAGCCATGCATAT
C9αmiR160A
E
Col-0
C9αmiR160A

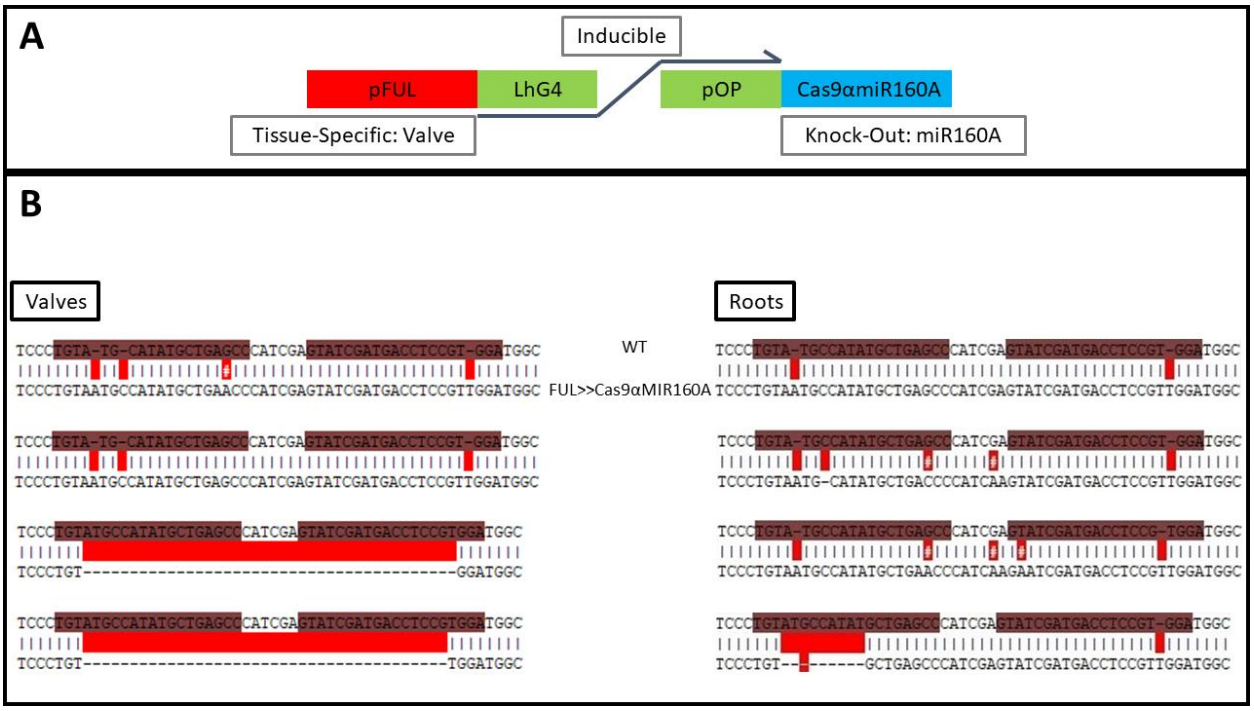
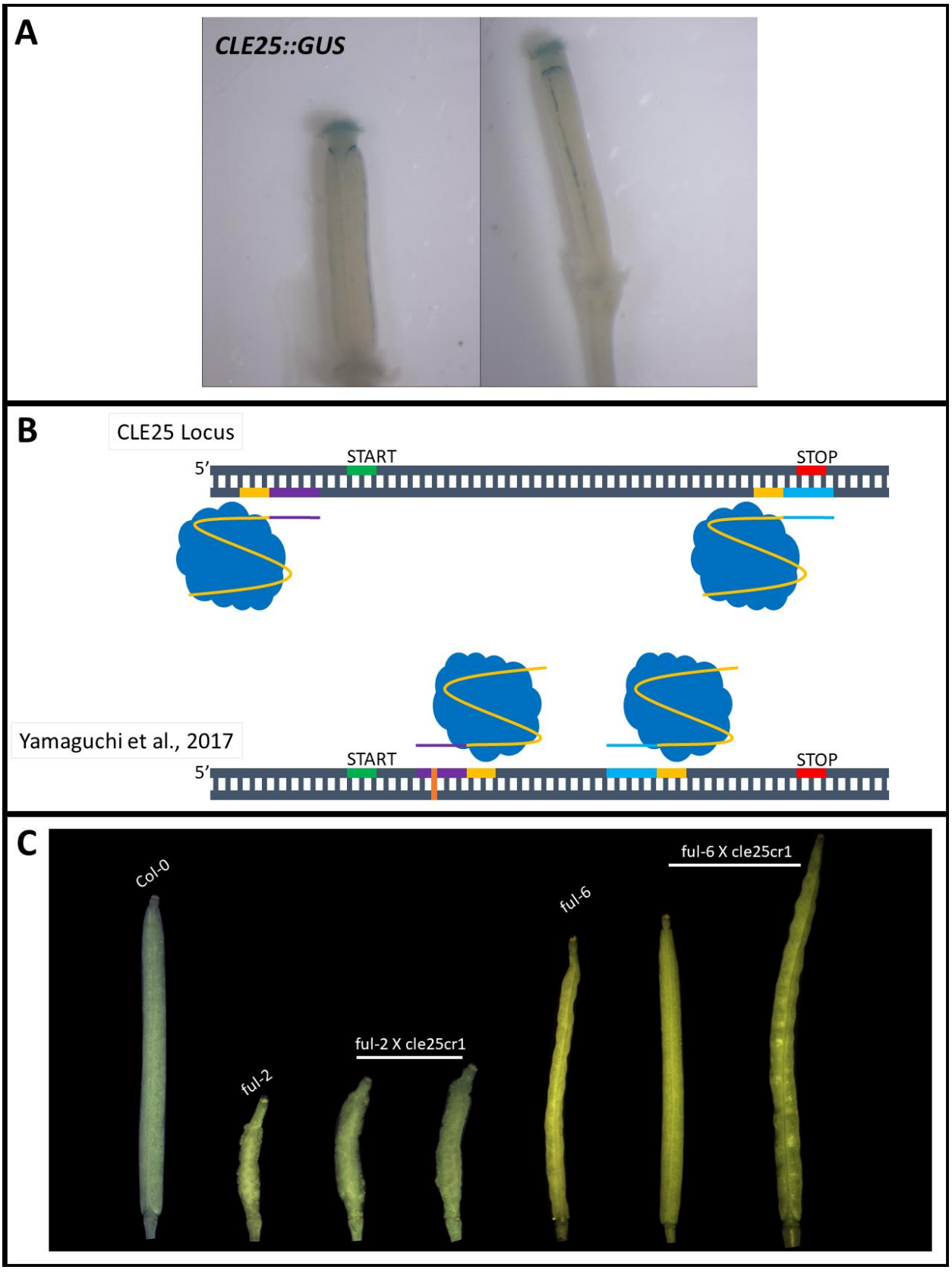


Figure 7. Using the pOP/LhG4 trans-activation system to induce tissue-specific Cas9-mediated gene knock-out.

(A) A model for the application of the pOP/LhG4 trans-activation system to induce valve-specific Cas9-mediated knock-out of *miR160A* function. **(B)** Comparison of sequencing results for the target region in the *miR160A* locus between C9α*miR160A* valve and root tissues.

Figure 8. *CLE25* knock-out in *ful* mutant background partially rescues *ful* mutant phenotype.

(A) Whole mount GUS staining of *CLE25* reporter in 2-week-old fruits. **(B)** Models showing gRNA targeting strategies in the *CLE25* locus for our *C9α**CLE25* (top) and *cle25cr1* (bottom) from Yamaguchi et al., 2017. Orange indicates the single nucleotide deletion generated by Yamaguchi et al., 2017. **(C)** Whole mount microscopic images of fruits for wild type Col-0, *ful-2*, two *ful-2 cle25cr1* double mutants, *ful-6*, and two *ful-6 cle25cr1* double mutants.


A
CLE25::GUS
B
CLE25 Locus
START
STOP
5′
Yamaguchi et al., 2017
START
STOP
5′
C
Col-0
ful-2
ful-2 X cle25cr1
ful-6
ful-6 X cle25cr1

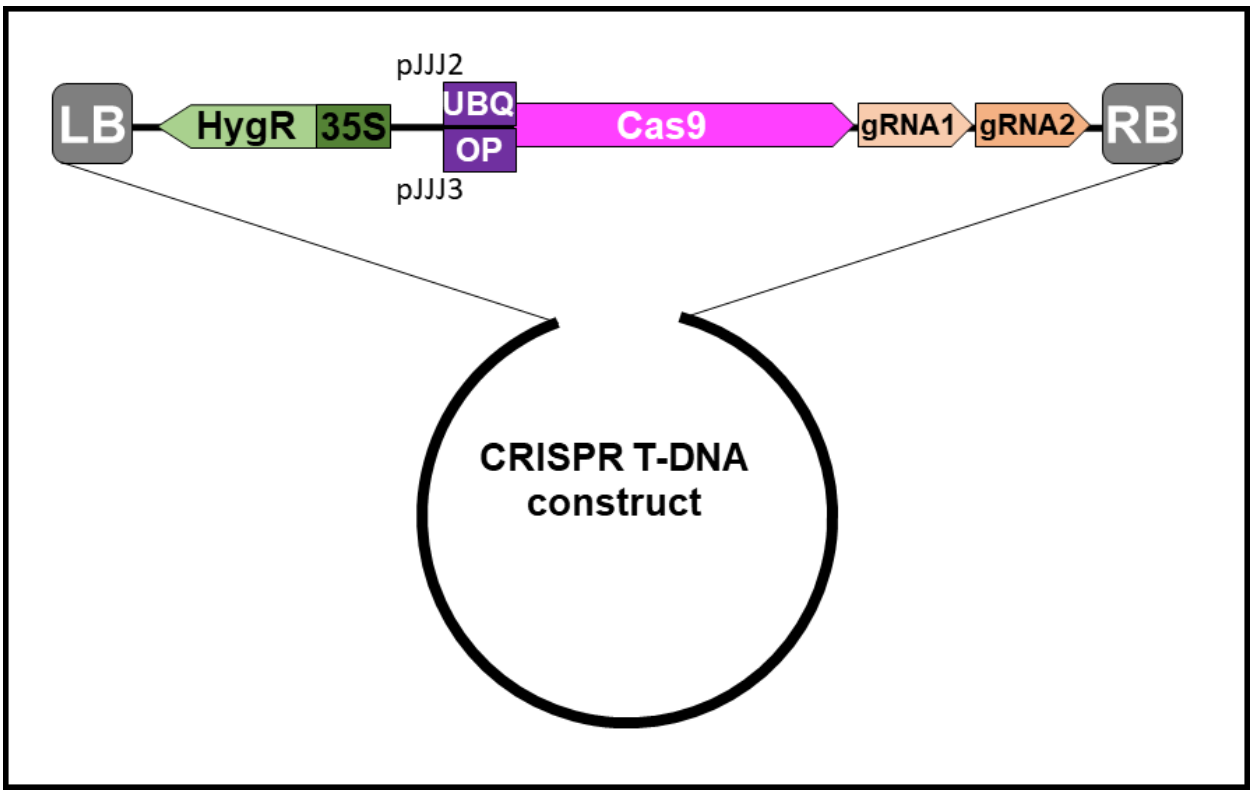


Figure 9. T-DNA vector construct used for delivering CRISPR/Cas9.

The design for the T-DNA vector used to deliver Cas9 mechanism. Two variants, pJJJ2 and pJJJ3, were created with different promoters, *UBQ* and *OP* respectively (Ripoll et al., unpublished).

APPENDIX B: TABLES AND TABLE LEGENDS

Table 1. Gene-specific gRNA sequences used to generate Cas9 knock-out mutants.

The gRNAs listed were cloned into the T-DNA vector pJJJ2 (or pJJJ3 in the case of *OP::C9*a*miR160A*).

T.A.I.R. = The Arabidopsis Information Resource

Gene	T.A.I.R. code	gRNA1 + PAM	gRNA2 + PAM	CRISPR Mutants
miR172D	AT3G55512	TCAGAAATCAGATTCTCTTA**TGG**	ATGCTGCAGCGGCAATTAAA**TGG**	*C9*a*miR172D*
miR160A	AT2G39175	TGTATGCCATATGCTGA**GCC**	GTATCGATGACCTCCGT**GGA**	*C9*a*miR160A, OP::C9*a*miR160A*
CLE25	AT3G28455	TACAGTCAATATCATACTCT**TGG**	GAGCAGAACATCCATGATGT**TGG**	*C9*a*CLE25*

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