Summer 8-12-2011

The Molecular Mechanism for Vegetative Phase Change: Regulation Ff Mir156 Expression and Action

Li Yang
liyang2@sas.upenn.edu

The Molecular Mechanism for Vegetative Phase Change: Regulation Ff Mir156 Expression and Action

Abstract
The timing of the transitions between the juvenile and adult vegetative stages (vegetative phase change) is important for shoot maturation in plants. The juvenile and adult vegetative stages are defined by a difference in reproductive competence (incompetent versus competent), but they are also associated with a variety of other morphological and physiological differences. An evolutionarily conserved microRNA, miR156, plays a central role in promoting the juvenile phase through its repression of ten adult-phase-inducing *SPL* family transcription factors. A decrease in miR156 abundance and a concomitant increase in *SPL* expression are correlated with the onset of adult traits. However, despite the importance of miR156 in regulating vegetative phase change, very little is known about the regulation of miR156 itself at either transcriptional or posttranscriptional levels. The aim of this work is to further the understanding of the factors that contribute to the regulation of miR156.

To identify the source of signals that repress miR156 and promote vegetative phase change, I performed organ ablation experiments in Arabidopsis. I discovered that defoliation, but not root or cotyledon ablation, delayed phase change, and this effect was attributable to an increase in the expression of *MIR156*. Defoliation also delayed phase change in *Nicotiana benthamiana, Zea mays* (maize), and *Acacia mangium*. Based on these results, I concluded that vegetative phase change is mediated by a leaf-derived signal that represses the transcription of MIR156. Furthermore, the possibility that sugar is the leaf signal was explored. Exogenous sugar repressed the expression of *MIR156*, resulting in an increase in *SPL* expression and early phase change. Consistent with this observation, mutants with reduced abundance of endogenous sugars had elevated miR156 expression and delayed phase change. This sugar response was dependent on the signaling function of the glucose sensor HXK1. To identify additional modifiers of the miR156 pathway, I performed a genetic screen using an SPL3-GFP translational reporter, identifying mutants that have either higher or lower GFP expression. This screen produced mutations in SUO, a BAH domain containing protein. SUO is a Processing-body (P-body) component and is specifically required for miR156-mediated translational repression, but not for miR156-mediated transcript cleavage. These results indicate that miR156-mediated translational repression plays an important role in regulating vegetative phase change.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Biology

First Advisor
R. Scott Poethig

Keywords
Arabidopsis, phase change, microRNA, SPL, miR156

Subject Categories
Biology | Botany | Developmental Biology | Genetics | Life Sciences | Molecular Genetics | Plant Biology | Plant Sciences

The molecular mechanism for vegetative phase change: regulation of miR156 expression and action

Li Yang

A DISSERTATION

in

Biology

Presented to the Faculties of the University of Pennsylvania in

Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2011

Supervisor of Dissertation

_____

R. Scott Poethig
Professor of Biology

Graduate Group Chairperson

_____

Paul D. Sniegowski
Associate Professor of Biology

Dissertation Committee

Dr. Nancy Bonini, Professor of Biology
Dr. Stephen DiNardo, Professor of Cell and Developmental Biology
Dr. Kimberly Gallagher, Assistant Professor of Biology
Dr. Meera V. Sundaram, Associate Professor of Genetics
Dr. Doris Wagner, Associate Professor of Biology

Acknowledgements

I would like to express my deepest gratitude to my advisor, Dr. R. Scott Poethig. This thesis would not have been possible without his “invisible” guidance. Scott is always the source of creative idea and insightful advice, but never imposes his opinion on my research, which allows me to enjoy the freedom when exploring science. He has set a perfect role model in my future career for his passion for science and enthusiasm in teaching.

Besides my advisor, I would like to thank the rest of my thesis committee for their encouragement and challenges.

I also want to thank my colleagues in the Poethig lab for their stimulating discussion and selfless sharing, especially, Dr. Matthew Willmann for his assistance in editing this thesis.

Finally, I owe my gratitude to my family for their endless love and support.

ABSTRACT

The molecular mechanism for vegetative phase change:

regulation of miR156 expression and action

Li Yang

R. Scott Poethig

The timing of the transitions between the juvenile and adult vegetative stages (vegetative phase change) is important for shoot maturation in plants. The juvenile and adult vegetative stages are defined by a difference in reproductive competence (incompetent versus competent), but they are also associated with a variety of other morphological and physiological differences. An evolutionarily conserved microRNA, miR156, plays a central role in promoting the juvenile phase through its repression of ten adult-phase-inducing *SPL* family transcription factors. A decrease in miR156 abundance and a concomitant increase in *SPL* expression are correlated with the onset of adult traits. However, despite the importance of miR156 in regulating vegetative phase change, very little is known about the regulation of miR156 itself at either transcriptional or posttranscriptional levels. The aim of this work is to further the understanding of the factors that contribute to the regulation of miR156.

To identify the source of signals that repress miR156 and promote vegetative phase change, I performed organ ablation experiments in Arabidopsis. I discovered that defoliation, but not root or cotyledon ablation, delayed phase change, and this effect was attributable to an increase in the expression of *MIR156*. Defoliation also delayed phase change in *Nicotiana benthamiana, Zea mays* (maize), and *Acacia mangium*. Based on these results, I concluded that vegetative phase change is mediated by a leaf-derived

signal that represses the transcription of MIR156. Furthermore, the possibility that sugar is the leaf signal was explored. Exogenous sugar repressed the expression of *MIR156*, resulting in an increase in *SPL* expression and early phase change. Consistent with this observation, mutants with reduced abundance of endogenous sugars had elevated miR156 expression and delayed phase change. This sugar response was dependent on the signaling function of the glucose sensor HXK1. To identify additional modifiers of the miR156 pathway, I performed a genetic screen using an SPL3-GFP translational reporter, identifying mutants that have either higher or lower GFP expression. This screen produced mutations in SUO, a BAH domain containing protein. SUO is a Processing-body (P-body) component and is specifically required for miR156-mediated translational repression, but not for miR156-mediated transcript cleavage. These results indicate that miR156-mediated translational repression plays an important role in regulating vegetative phase change.

Table of Contents

List of Tables

List of Figures

1. INTRODUCTION

1.1. What is vegetative phase change?

In the sporophytic generation, plants display several important developmental stages, namely embryonic, juvenile vegetative, adult vegetative and reproductive stages. **Vegetative phase change** (hereafter referred to as phase change) is the transition between the juvenile and adult vegetative stages (Brink, 1962; Allsopp, 1967a; Poethig, 1990).

Although it is widely accepted that distinctive stages exist during vegetative development, the exact demarcation between the juvenile and the adult phases is still vague (Jones, 1999). Goebel first differentiated the juvenile and adult phases by a difference in reproduction capacity (Goebel, 1900). Since then, reproductive competence has been considered a consistent distinction between these phases (Doorenbos, 1954; Allsopp, 1967a; Poethig, 1990). However, determining reproductive competence is not always straightforward. Reproductive competence in flowering plants is defined as the ability to respond to floral inducing signals. Research in Arabidopsis has demonstrated that several distinct, overlapping pathways control flowering time as a response to intrinsic (hormones) and extrinsic (photoperiod, temperature) signals (Levy and Dean, 1998; Mouradov et al., 2002; Komeda, 2004; Amasino, 2010). This variability makes it difficult to define inductive conditions and more difficult to define reproductive capacity. In addition, some *Eucalyptus* species flower on juvenile branches, suggesting that reproductive competence and the onset of the adult phase can be separated (Wiltshire et

al., 1991). These observations are consistent with the discovery that some mutants affecting vegetative phase change in Arabidopsis (e.g. *zippy*) do not alter flowering time (Hunter et al., 2003), and key mutants affecting flowering time do not affect the timing of vegetative phase change (Willmann and Poethig, 2011). Although reproductive competence is a consistent hallmark of phase change, phase change and flowering are regulated by two distinct pathways.

Another major readout of vegetative phase change is heteroblastic development. The term heteroblasty describes developmental variation in leaf morphology (or leaf-like organs) produced during shoot maturation (Figure 1.1) (Goebel, 1900). A classic example of heteroblastic development is by *Acacia spp.,* which produces pinnate leaves in the juvenile stage and simple leaves called phyllodes in the adult stage (Goebel, 1900). Genetic screens using heteroblastic traits in maize and Arabidopsis have revealed common regulatory pathways that control the timing of phase change (see 1.4), demonstrating that these traits are reliable markers of vegetative phase change (Poethig, 2009). However, not all plants generate two distinct types of leaves that can be easily classified into juvenile and adult forms. For example, *Pseudopanax crassifolius* produces 8 different types of leaves. These leaves vary in shape, cuticle characters, and histological structure (Gould, 1993). In addition, Goebel described homoblastic plants in which no leaf variation was observed on successive nodes (Goebel, 1900). In these cases, leaf morphology is not an ideal marker for demarcating the juvenile and the adult phases.

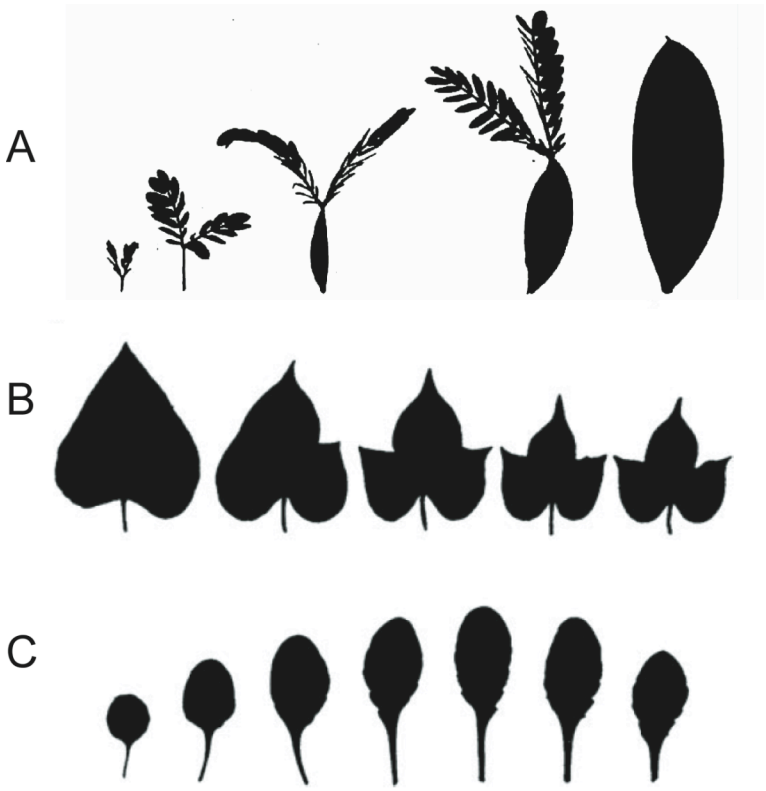


Figure 1.1 Examples of heteroblastic development

A) The first five leaves of *Acacia Mangium*. The first two leaves are pinnate. The late-formed leaves switch to phyllodes. B) The first five leaves of *Ipomea caerulea*. The leaf shape changes from simple leaf to lobed leaf. C) Leaves two through eight in the Columbia ecotype of *Arabidopsis thaliana*. B and C redrawn from (Kerstetter and Poethig, 1998).

Beyond reproductive competence, leaf shape and size, a number of other morphological or physiological traits may vary between the juvenile and adult phases. The traits that distinguish these two developmental stages are specific to each species, but may include differences in adventitious rooting ability, epidermal cell size, cuticle thickness, the presence or absence of epidermal hairs, wood quality, the production of secondary metabolites, and disease resistance (Brink, 1962; Poethig, 1990).

However, such traits have a clear limitation for use as phase specific markers. First, plant physiology and morphology vary from species to species. It is not surprising that good markers in one species may not exist in other species, considering the dramatic differences in their life history. Second, parallel developmental programs interact with the phase change program to simultaneously influence all of these traits. Such complexity makes it difficult to distinguish a phase specific change from the changes induced by other regulatory pathways.

1.2.The role of miR156 and the *SPL* genes in regulating phase change

Due to the limitations of morphological and physiological markers, the identification of a reliable, general and sensitive molecular marker for phase change is crucial for further understanding this process. The microRNA (miRNA) miR156 has recently emerged as the long-awaited molecular marker for phase change (Poethig, 2009).

miR156 belongs to a class of 20-22 nucleotide microRNAs, which down-regulate gene expression either by translational repression or mRNA cleavage via complementary

base-pairing (Reinhart et al., 2002; Rhoades et al., 2002). In plants, primary-miRNAs are transcribed by RNA polymerase II, like mRNAs, and are associated with a cap-binding complex containing ABISICIC ACID HYPERSENSITIVE (ABH1). Primary-miRNAs are processed into miRNA/miRNA* duplexes by a protein complex containing DICER-LIKE1 (DCL1), HYPONASTIC LEAVES1 (HYL1), and SERRATE (SE). Both strands of the duplex are then methylated on their 3'-ends by HUA ENHANCER (HEN1), and transported from nucleus into the cytoplasm by HASTY (HST), an ortholog of mammalian Exportin5. In cytoplasm, the miRNA but not the miRNA* is incorporated into the RNA Induced Silencing Complex (RISC), where it directs mRNA cleavage or translational repression. As a core component of the RISC complex, ARGONAUTE1 (AGO1) can bind to the small RNA and enzymatically cleave their complementary mRNAs (Voinnet, 2009). Because of its important role in miRNA-directed silencing, the function and stability of AGO1 is highly regulated (Jones et al., 2006; Vaucheret et al., 2006; Zhang et al., 2006; Csorba et al., 2010; Earley et al., 2010). Plant cyclophilin40, also known as SQUINT (SQN), was found to promote AGO1 activity (Smith et al., 2009).

Defects in miRNA biogenesis usually cause precocious phase change. *hst* and *sqn* were originally isolated for their early phase change phenotype. Both of these mutations cause an early onset of abaxial trichomes, produce leaves that are longer and serrated than in wild type (Berardini et al., 2001; Bollman et al., 2003). Several hypomorphic *ago1* alleles were also isolated in genetic screens for early phase change mutants. These *ago1* mutations phenocopy *sqn* in leaf shape, trichome distribution, rate of leaf initiation and phyllotaxy (Berardini et al., 2001; Smith et al., 2009). These observations imply that

one or more miRNAs are required to promote the juvenile phase or to repress the onset of the adult phase.

Subsequent studies revealed that miR156 is an important regulator of phase change. miR156 is encoded by 8 loci (*MIR156A-MIR156G*) in the Arabidopsis genome (Figure 1.2 A) (Xie et al., 2005). The temporal expression pattern of miR156 was first discovered by comparing the miRNA levels of wild type and the *hst* plants (Park et al., 2005). Park et al. (2005) noticed that miR156 highly expressed in immature rosette leaves compared to mature rosette leaves (Park et al., 2005). Wu et al. (2006) further showed that the accumulation of mature miR156 was temporally regulated. The mature form of miR156 accumulated in young seedlings, and the level decreased dramatically upon the onset of the adult stage (Wu and Poethig, 2006). Over-expressing *MIR156A* under the constitutive 35S promoter significantly delayed the transition from the juvenile to the adult stage: these plants produced more juvenile leaves and exhibited a delay in flowering (Schwab et al., 2005; Wu and Poethig, 2006) (Figure 1.2 B). Furthermore, over-expressing *MIR156A* suppressed most of the precocious phenotypes of *sqn*, suggesting that the early phase change phenotype of *sqn* is due to impaired miR156 function (Smith et al., 2009). On the other hand, blocking the normal function of miR156 using target mimicking (*35S::MIM156*) resulted in precocious phase change. The first two leaves of plant expressing *35S::MIM156* are highly serrated and elongated, and have a short petiole, and these plants flowers with fewer leaves than wild type (Wu and Poethig, 2006; Franco-Zorrilla et al., 2007) (Figure 1.2 B). Importantly, altering the level or activity of miR156 affects multiple phase-related traits, indicating that miR156 is an upstream regulator of all of these phenotypes (Schwab et al., 2005; Wu and Poethig, 2006).

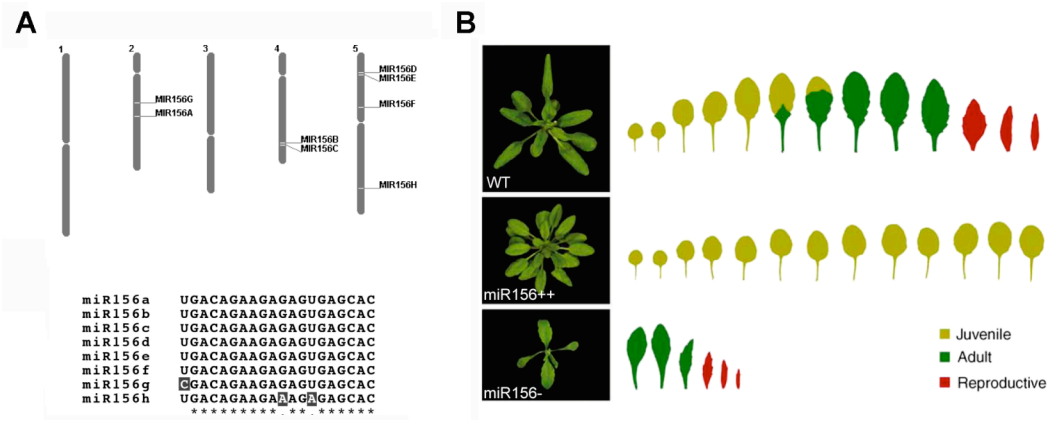


Figure 1.2 miR156 regulates phase change

A) The chromosome view of *MIR156* precursors in the Arabidopsis genome. B) Functional significance of miR156 in Arabidopsis. Overexpression of miR156 (miR156++) prolongs the juvenile phase, while blocking miR156 function (miR156-) causes precocious phase change (Poethig, 2009).

miR156 targets ten members of the *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) gene family, each of which specifies different subsets of adult traits (Figure 1.3) (Reinhart et al., 2002; Rhoades et al., 2002). *SPL* genes are plant specific transcriptional factors and are highly conserved from bryophytes to angiosperms (Klein et al., 1996; Riese et al., 2007; Guo et al., 2008). Sixteen *SPL* genes in the Arabidopsis genome share a conserved SBP domain that enables them to enter the nucleus and interact with a common DNA motif characterized by a core palindrome sequence of GTAC (Cardon et al., 1999; Birkenbihl et al., 2005). 10 *SPL* genes have miR156 binding sites either in the coding region or the 3'-UTR. They can be further classified into four taxonomic subgroups: *SPL3*/*SPL4*/*SPL5*, *SPL9*/*SPL15*, *SPL2*/*SPL10*/*SPL11* and *SPL6*/*SPL13* (Figure 1.3). Among them, the transcripts of *SPL3*/*SPL4*/*SPL5* increase during shoot maturation, which is complementary to the expression pattern of miR156 (Cardon et al., 1997; Cardon et al., 1999; Wu and Poethig, 2006; Gandikota et al., 2007). Although the *SPL9* transcript remains constant during shoot maturation, a translational reporter shows that SPL9 protein accumulates predominantly in the adult phase (unpublished data). The high expression of *SPL* genes in adult stage suggests that their roles are to specify adult traits or inhibit juvenility.

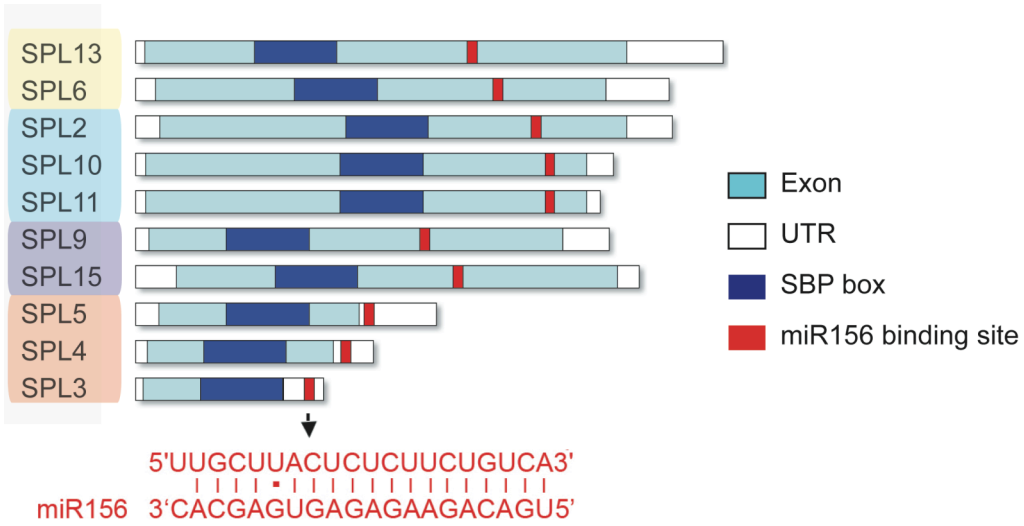


Figure 1.3 miR156 targets *SPL* genes
miR156 targets 10 *SPL* genes. The miR156-binding sites (red) in *SPL3/SPL4/SPL5* locate in the 3'-UTR. Other *SPL*s have the binding site in coding region. The dark-blue box indicates conserved SBP box.

The divergent functions of the *SPL* genes help to explain how miR156 regulates the expression of multiple adult traits (Figure 1.4 B). Multiple *SPL* genes regulate heteroblastic development in Arabidopsis. Expressing *SPL3, SPL4, SPL5, SPL9, SPL10, SPL11* without the miR156 binding site caused the precocious appearance of abaxial trichomes, and in some cases produced adult leaf shape as well (Wu and Poethig, 2006; Wu et al., 2009). Conversely, loss-of-function mutations of *spl9* slightly delays phase change, as measured by the onset of abaxial trichomes.(Schwarz et al., 2008; Wu et al., 2009). Such delay was enhanced when combined with *spl15* mutations, indicating that these genes act redundantly in regulating the appearance of the abaxial trichomes (Wu et al., 2009). The molecular mechanism of how *SPL* genes control the appearance of abaxial trichomes is not yet clear. Yu et al. (2010) reported that *SPL9* directly activates the transcription of *TRICHOMELESS1* (*TCL1)* and *TRIPTYCHON* (*TRY*), two negative regulators of trichome development, which is important for the temporal decrease of trichome density along the stem (Yu et al., 2010). Although the density of trichomes increases on the abaxial surface of leaves (opposite of its temporal change on the stems), *SPL* genes may promote some activators of trichome development in a temporal manner during leaf development. Another possibility is that *SPL* genes interact with the leaf polarity pathway, because the loss-of-function mutations of the abaxial-promoting gene *KANADI* causes an early trichome onset phenotype (Kerstetter et al., 2001). In addition to their redundant roles, *SPL* genes have distinct functions of controlling leaf shape. Over-expressing *SPL3, SPL4, SPL5* with or without the miR156 binding site did not change leaf shape (Wu and Poethig, 2006). On the other hand, expressing resistant forms of *SPL9*, *SPL10*, *SPL11*, *SPL15* dramatically altered leaf shape, converting the round and

smooth-margined juvenile leaves into elongated and serrated adult leaves (Wang et al., 2008; Shikata et al., 2009; Wu et al., 2009). Although an *spl3 spl4 spl5* triple mutant has not been identified, it is unlikely that it will affect leaf shape considering he absence of gain-of-function phenotype.

Another heteroblastic trait regulated by the *SPL* genes is the temporal change in cell size on leaves. In 1904, Zalenski reported that the average size of leaf cells decreased from the base to the tip of the shoot (Zalenski, 1904). Usami et al. (2009) also observed this phenomenon in Arabidopsis. In a screen to identify the pathways governing leaf size and shape, they isolated a dominant allele of *SPL15* (*msc1-D*/*spl15-1D*) that contained a mutation in the miR156-binding site. *spl15-1D* leaves had increased cell number and decreased cell size as well as early abaxial trichomes. Plants over-expressing the resistant forms of the *SPL3*, *SPL4*, *SPL5*, also had smaller cell size in the first leaf, suggesting that the SPL3 subgroup also controls the phase-regulated change in cell size (Usami et al., 2009). Thus, the *SPL* genes are responsible for the temporal regulation of cell size.

SPL genes regulate flowering time in at least three different ways (Cardon et al., 1997; Gandikota et al., 2007; Wang et al., 2009b; Wu et al., 2009; Yamaguchi et al., 2009). *SPL3* directly activates the transcription of *FUL*, *AP1* and *LFY* (Yamaguchi et al., 2009), while *SPL9* targets *SOC1* and *AGL42* (Wang et al., 2009b). In addition to the direct activation of floral inducers, *SPL9*, *SPL10* also promote flowering in an indirect way. *SPL9*, *SPL10* repress the floral repressors *TOE1* and *TOE2* by increasing the expression of *MIR172*, whose miRNA targets several AP2-like genes, including *TOE1* and *TOE2* (Wu et al., 2009). It is interesting to note that multiple *SPL* genes control flowering time through different pathways, which can be interpreted as a insurance

mechanism to prevent plants from flowering in the juvenile phase.

The function of *SPL* (*SBP*) genes is summarized in Table 1.

1.3. A conserved role for the miR156-SPL pathway in regulating phase change

The sequence and function of miR156 and the *SPL* genes are highly conserved in the plant kingdom (Axtell et al., 2007; Barakat et al., 2007; Guo et al., 2008; Willmann and Poethig, 2007). The miR156 pathway in maize is similar to that in Arabidopsis. *corngrass1* is a dominant mutant with a prolonged juvenile phase. The phenotype was due to mutations in the regulatory region of a gene that encodes both *zma-MIR156B* and *zma-MIR156C*, and causes over-expression of this gene (Chuck et al., 2007). In *Cg1* mutants, both miR172 and *tga1* (*teosinte glume architecture1*), an SBP gene involved in maize domestication, are down-regulated (Chuck et al., 2007). *Tp1* and *Tp2* are two other semi-dominant mutants in maize with a prolonged juvenile phase (Poethig, 1988a; Poethig, 1988c; Bassiri et al., 1992; Dudley and Poethig, 1993). miR156 is also over-expressed in these mutants (Park and Poethig, unpublished). Genetic analysis demonstrates that *glossy15*, a mutant with precocious phase change, acts downstream of *cg1*, *tp1* and *tp2*, and only specifies leaf epidermal cell identity, including the presence of epicuticular waxes and leaf hairs as well as epidermal cell wall characteristics (Evans et al., 1994; Moose and Sisco, 1994b). The *GL15* gene encodes an AP2-like transcriptional factor that is targeted by miR172 (Moose and Sisco, 1996; Lauter et al., 2005). The regulatory hierarchies for phase change in Arabidopsis and maize is very similar: miR156 promotes juvenile traits by repressing the *SPL* (*SBP*) genes and miR172.

Table 1: A summary of *SPL* (*SBP*) gene function

Species	Gene	Function	MiR156 targeted	Reference
Arabidopsis Thaliana	*AtSPL2*	lateral organ morphogenesis in reproductive stage, anther development	Yes	(Shikata et al., 2009; Xing et al., 2010)
	AtSPL3	vegetative phase change, flowering time, trichome distribution	Yes	(Cardon et al., 1997; Wu and Poethig, 2006; Gandikota et al., 2007;Yamaguchi et al., 2009; Yu et al., 2010; Jung et al., 2011)
	AtSPL4	vegetative phase change, flowering time	Yes	(Wu and Poethig, 2006; Wu et al., 2009)
	AtSPL5	vegetative phase change, flowering time	Yes	(Wu and Poethig, 2006; Wu et al., 2009)
	AtSPL7	copper homeostasis	No	(Kropat et al., 2005; Yamasaki et al., 2009)
	AtSPL8	pollen sac development, GA-mediated anther development, anther development	No	(Unte et al., 2003; Zhang et al., 2007; Xing et al., 2010)
	AtSPL9	phase change, flowering time, plastochrone, trichome distribution, anther development	Yes	(Schwarz et al., 2008; Wang et al., 2009b; Wu et al., 2009; Xing et al., 2010; Yu et al., 2010)
	AtSPL10	embryogenesis, vegetative phase change, lateral organ morphogenesis in reproductive stage	Yes	(Shikata et al., 2009; Wu et al., 2009; Nodine and Bartel, 2010; Yu et al., 2010)
	AtSPL11	embryogenesis, lateral organ morphogenesis in reproductive stage	Yes	(Shikata et al., 2009; Nodine and Bartel, 2010)
	AtSPL13	trichome distribution, post-germination switch	Yes	(Martin et al., 2010a, b; Yu et al., 2010)
	AtSPL14	leaf development, sensitivity to fumonisin B1	No	(Stone et al., 2005)
	AtSPL15	phase change, flowering time, plastochrone, cell size in leaf, anther development	Yes	(Schwarz et al., 2008; Usami et al., 2009; Xing et al., 2010)
Oryza sativa	*OsSPL8/OsLG1*	leaf morphogenesis, laminar joint, auricle and ligule development	No	(Lee et al., 2007)
	OsSPL14	rice architecture	yes	
	OsSPL14/WFP	panicle branching, grain yield	yes	(Miura et al., 2010)
Antirrhinum majus	*AmSBP1*	flowering time	?	(Preston and Hileman, 2010)
Lycopersicon esculentum	*LeSPL-CNR*	fruit ripening	Yes	(Manning et al., 2006)
Eucalyptus globulus	*EglSPL3*	vegetative phase change	Yes	(Wang et al., 2011)
	EglSPL9	vegetative phase change	Yes	(Wang et al., 2011)
Populus x canadensis	*PcSPL3*	vegetative phase change	Yes	(Wang et al., 2011)
	PcSPL9	vegetative phase change	Yes	(Wang et al., 2011)
Zea mays	*TSH4*	bract development, establishing meristem boundaries.	Yes	(Chuck et al., 2010)
	TGA	grain architecture	Yes	(Chuck et al., 2007)
	LG1	ligules and auricles development, axial patterning in leaf	?	(Moreno et al., 1997; Foster et al., 2004)
Physcomitrella patens	*PpSBP1*	side branch formation	?	(Riese et al., 2008)
	PpSBP4	side branch formation	?	(Riese et al., 2008)

miR172 further represses AP2-like genes to specify epidermal cell identity.

miR156 is one of the few miRNAs that is conserved from bryophytes (e.g. *Physcomitrella patens*) to angiosperms (Arabidopsis and maize) (Arazi et al., 2005; Talmor-Neiman et al., 2006; Axtell et al., 2007; Barakat et al., 2007). *SBP* genes also exist in *Physcomitrella patens*, and the cleavage products of *PpSBP*s by miR156 have been identified, suggesting a conserved repressive role of miR156 on these *SBP* genes (Arazi et al., 2005; Axtell et al., 2007). Not only the sequences but also the function of miR156 seems to be conserved across different plant species. miR156 regulates a similar set of traits in Arabidopsis, maize, *Oryza sativa* (rice) and *Lycopersicon esculentum* (tomato). Altering miR156 expression in these species affects the expression of juvenile characteristics, the length of plastochron, branching, rooting ability, flowering time and floral structure (Wu and Poethig, 2006; Chuck et al., 2007; Gandikota et al., 2007; Schwarz et al., 2008; Wang et al., 2008; Wang et al., 2009b; Wu et al., 2009; Miura et al., 2010; Zhang et al., 2011). A conservation of miR156 function has been further demonstrated in tree species. In *Acacia*, *Eucalyptus, Populus* and *Hedera. helix,* high expression of miR156 was always associated with juvenile traits, and with a low level of miR172 and *SPL* genes; furthermore, over-expression of miR156 in *Populus* causes many of the same phenotypes observed in *Arabidopsis* and maize (Wang et al., 2011). These observations demonstrate that vegetative phase change in woody and herbaceous plants is regulated by the same mechanism, and that miR156 is an evolutionarily conserved molecular marker for this process.

1.4. A model for the regulation of phase change by miR156

In his review on phase change, Sussex (1976) raised an important question: "How is the large number of genes that must regulate the expression of a specific phase turned on or off coordinately?" (Sussex, 1976). The identification of miR156 and the further demonstration of its functions provide the long-awaited molecular basis for phase change. Post-transcriptional regulation by miRNAs is an efficient way to coordinately turn off a set of transcripts that possess a sequence complementary to these miRNAs (Figure 1.4 A, B). In *C. elegans*, a similar mechanism is used in heterochronic control of cell fate (Ambros, 2000; Pasquinelli and Ruvkun, 2002). By adding or deleting the complementary sequence of miR156 in a gene, plants can recruit or expel a certain feature as a phase-specific trait. *SPL* genes are among the genes that regulate the expression of various traits in the adult phase. Interestingly and importantly, these *SPL* genes have overlapping and distinct roles in specifying adult features. For example, *SPL9*, *SPL10*, *SPL11* specify leaf shape; *SPL3*, *SPL4*, *SPL5* specify flowering competence. The divergent roles of *SPL* genes partly explain the complexity of traits associated with vegetative phase change (Figure 1.4 B).

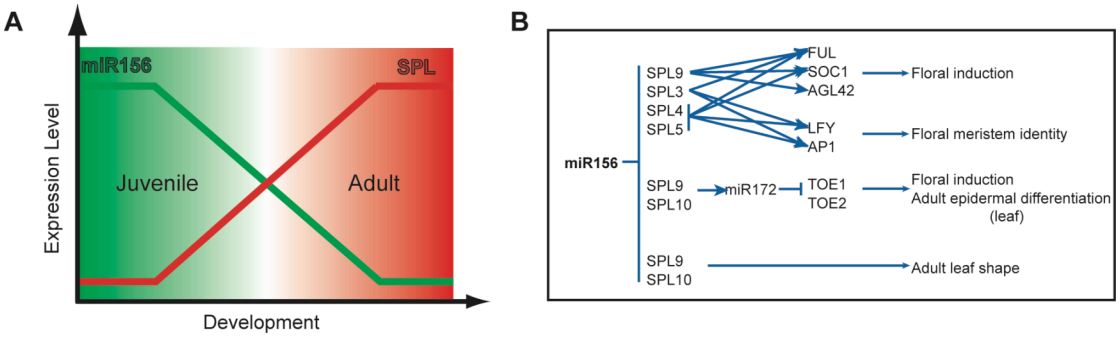


Figure 1.4 The temporal expression of miR156 and SPL genes controls phase specific traits.

A) A model illustrating the temporal expression of miR156 and SPL genes. The expression of miR156 remains high during the juvenile phase. The drop of miR156 is associated with the increase of SPL genes. In this model, the juvenile phase is defined by a high level of miR156, while the SPL genes specify the adult traits. B) The redundant and distinct function of SPL genes in control adult phenotype. (B) is from (Poethig, 2009).

1.5.Questions

The mechanism by which plants measure developmental time is an important question for understanding vegetative phase change. All phase related traits switch in a predictable fashion under certain growth conditions. Because miR156 is necessary and sufficient to shift the appearance of these traits, this question can be rephrased "how is miR156 temporally regulated?". Despite the important role of miR156 in phase change, little is known about the regulation of its temporal expression or action. As pointed out by Poethig (2010), "The temporal decrease in miR156 expression is of the crucial importance, and until the mechanism of this event is known our understanding of vegetative phase change will remain juvenile. (Poethig, 2010)"

In this thesis, I will address three related questions:

1) What is the source of the factors that regulate vegetative phase change?

2) What is the nature of these factors and how do they regulate miR156?

3) What are the unknown modifiers of the miR156-SPL pathway?

2. A LEAF-DERIVED SIGNAL PROMOTES PHASE CHANGE BY REPRESSING THE TRANSCRIPTION OF MIR156

(Related work is published on Development. 2011 Jan;138(2):245-9)

2.1. Abstract

Vegetative phase change in Arabidopsis is regulated by miR156, a microRNA that promotes the expression of the juvenile phase and represses the expression of the adult phase. miR156 is expressed at a very high level early in shoot development and then decreases, leading to the onset of the adult phase. To determine the source of the factors that regulate vegetative phase change, we examined the effect of root and leaf ablation on the timing of this transition. Ablation of the root system or cotyledons had no effect on the timing of vegetative phase change, but ablation of leaf primordia delayed this transition in a miR156-dependent fashion. This treatment produced an increase in the overall abundance of miR156—which was attributable to an increase in the transcription of some, but not all, of the miR156 genes in Arabidopsis—and decreased the expression of *SPL* genes regulated by miR156. miR156 levels were also elevated by leaf ablation in *Nicotiana benthamiana* and *Acacia Mangium,* and in rejuvenating shoot apices of maize cultured in vitro. We conclude that vegetative phase change is initiated by a signal(s) produced by leaf primordia, which acts by repressing the transcription of specific members of miR156 gene family.

2.2. Background

The shoot apex of plants produces different types of leaves, buds, and internodes at different times during development. Some features change continuously, but others change in a more abrupt fashion, allowing shoot development to be divided into discrete juvenile, adult, and reproductive phases (Poethig, 2003). The onset of the reproductive phase (floral induction) has been intensively studied for many decades and now quite well understood (Amasino, 2010; Fornara et al., 2010). Much less is known about the mechanism of the juvenile-to-adult transition (vegetative phase change), but recent studies in Arabidopsis and maize have begun to reveal some of the genes that regulate this transition. The most important of these is the microRNA, miR156. In both Arabidopis (Wu and Poethig, 2006) and maize (Chuck et al., 2007), miR156 is expressed at high levels during the juvenile stage, and drops dramatically upon the transition to the adult stage. Constitutive expression of miR156 prolongs the expression of the juvenile phase whereas a reduction in miR156 activity accelerates vegetative phase change, indicating that miR156 is a key regulator of this transition (Wu and Poethig, 2006; Chuck et al., 2007; Wu et al., 2009).

The source of the signal(s) that regulate vegetative phase change is unknown. It has long been suspected that vegetative phase change is regulated by factors that originate outside the shoot apex because the expression of juvenile traits can be prolonged by heavy pruning (Schaffalitzky de Muckadell, 1954) and defoliation (Ashby, 1948; Njoku, 1956b). Furthermore, culturing shoot apices in vitro often results in their rejuvenation (Mullins et al., 1979; Monteuuis and Bon, 1989; Brand and Lineberger,

1992; Irish and Karlen, 1998). Although these experiments suggest that leaves are the source of a factor that promotes the adult phase, they do not eliminate the possibility that vegetative phase changes is regulated by the root system. Indeed, the observation that adult ivy can be rejuvenated by grafting shoots to juvenile root stocks (Doorenbos, 1954; Stoutemyer and Britt, 1961), or by co-culturing the adult shoots with root-producing juvenile stocks (Frank and Renner, 1956), suggests that the root system may play an important role in this phenomenon. Several other woody species can also be regenerated by sequential grafting to juvenile root stocks (Huang et al., 1992; Fraga et al., 2003; Husen and Pal, 2003; Moon et al., 2008).

A major source of confusion in all of these studies is that different traits are used as markers of vegetative phase change in different species. Many things change during shoot development, and it is difficult to know if any particular trait is regulated by the program that controls vegetative phase change or by some other change in shoot physiology. In woody plants these possibilities are operationally distinguished by the stability of the trait: traits that are stably expressed in grafts or re-rooted shoots are thought to be components of an ontogenetic program of shoot maturation (vegetative phase change), whereas those that are readily reversed by these treatments are attributed to age-related changes in the physiology or size of the shoot (Wareing, 1959; Fortanier and Jonkers, 1976; Day et al., 2002). However, this definition is not universally applicable because traits that are widely accepted as being phase-specific (e.g. rooting ability) can be modified by grafting, and there is no evidence that juvenile and adult phases of shoot development are stably expressed in herbaceous plants.

In both maize and Arabidopsis, juvenile and adult phases of shoot development are defined by sets of traits that change in coordinated fashion during shoot development (Poethig, 1988b; Evans et al., 1994; Moose and Sisco, 1994b; Chien and Sussex, 1996a; Telfer et al., 1997). The identification of genes that control the expression of these traits now makes it possible to define these phases molecularly, based on the expression level of miR156 and the genes it regulates (Schwab et al., 2005; Wu and Poethig, 2006; Chuck et al., 2007; Strable et al., 2008; Wu et al., 2009). More importantly, because miR156 is both necessary and sufficient for vegetative phase change (Wu et al., 2009), it is now possible to identify the factors that regulate the timing of this transition by determining their effect on the expression of miR156.

We investigated the role of the root system and leaf primordia in vegetative phase change by ablating these organs at early stages of Arabidopsis development. Our results indicate that the root system plays little or no role in vegetative phase change, whereas leaves promote this transition. We show that the affect of leaf ablation on vegetative phase change requires the activity of miR156, and that this treatment causes a significant increase in miR156 in Arabidopsis, maize, *Nicotiana benthmiana* and *Acacia Mangium*. We conclude that vegetative phase change is mediated by a factor(s) produced by leaf primordia that acts by repressing the expression of miR156.

2.3.Results

2.3.1. Roots are not required for vegetative phase change

To investigate the role of the root system in vegetative phase change in Arabidopsis, we generated rootless plants using a transgenic approach. For this purpose, we took advantage of an enhancer trap line (E1735) that expresses the yeast transcriptional activator GAL4 in the quiescent cells of the embryonic and post-embryonic root apical meristem, starting at the heart stage of embryogenesis (Figure 2.1 A, B). E1735 was crossed to a line heterozygous for a transgenic construct in which the alpha chain of diptheria toxin (DTA) is fused the GAL4 promoter (UAS::DTA). The F1 progeny from this cross segregated phenotypically normal plants, as well seedlings displaying varying degrees of hypocotyl and root formation (Figure 2.1 C, D). The most severely affected seedlings had a very short hypocotyl and no visible root system; these severely affected plants failed to express the GFP reporter present in E1735, indicating that they completely lacked root cells (Figure 2.1 C, D). On MS medium supplied with 1% sucrose these rootless plants produced abnormally small but viable rosettes, and eventually flowered (Figure 2.1 E). In Arabidopsis, juvenile leaves lack trichomes on the abaxial surface of the leaf blade, whereas adult leaves possess abaxial trichomes (Chien and Sussex, 1996a; Telfer et al., 1997). Despite their difference in size, there was no significant difference between the number of leaves lacking abaxial trichomes (juvenile

leaves) in plants with, and without roots (Figure 2.1 E). We conclude that the root system does not play a significant role in vegetative phase change in Arabidopsis.

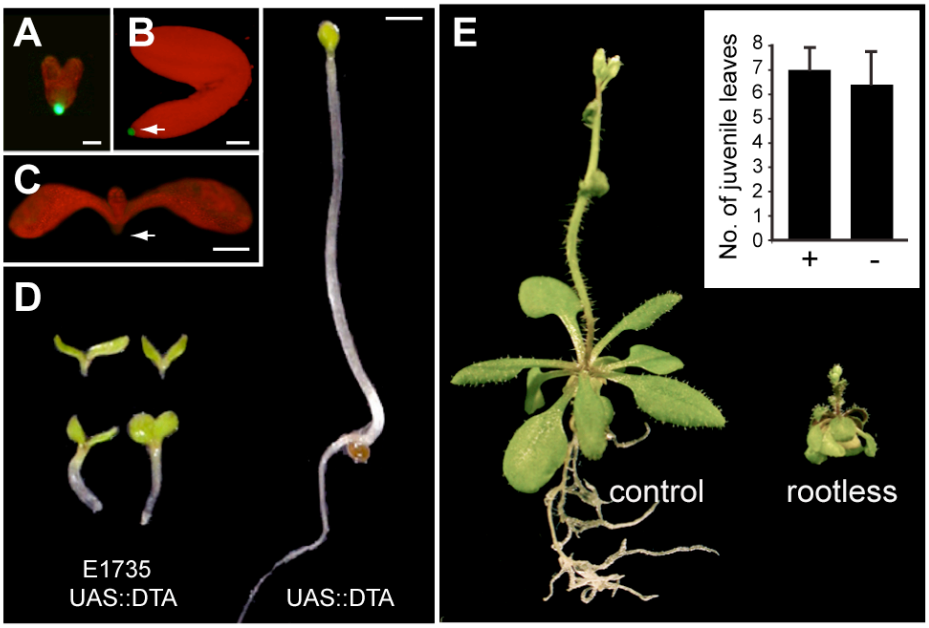


Figure 2.1: Roots are not required for vegetative phase change.

A) Heart stage and B) bent cotyledon stage E1735 embryos expressing GFP in the root apical meristem. C) Rootless E1735/+ UAS::DTA/+ seedling. This seedling does not express GFP in the position of the root tip (arrowhead). D) UAS::DTA/+ seedling, and the rootless progeny from a cross of this line to E1735 . E) Rosette morphology and the number of leaves without abaxial trichomes (juvenile leaves) of sibling E1735/+ and E1735/+ UAS::DTA/+ plants. The control plant in (E) is E1735/+ UAS::DTA/+. n=10. P>0.05. Scale bar = 20 μm in A & B, 1 mm in C, 2 mm in D.

2.3.2. Leaf ablation delays phase change by increasing the expression of miR156

We then examined if cotyledons or leaves regulate vegetative phase change by removing these organs at various times after germination. Removing cotyledons from 7-day-old seedlings delayed their growth significantly, and produced a slight delay in the production of abaxial trichomes (Figure 2.2 A, B, C), but later treatments had no significant effect on either growth rate or abaxial trichome production (Figure 2.2 C). In contrast, ablating the first two leaf primordia produced leaves at a slightly faster rate than untreated and wounded controls (Figure 2.3 A, B, C), and exhibited a marked delay in expression of several phase-specific leaf traits (Figure 2.3 D, E, F). The abaxial trichome production was delayed by one or two plastochrons, when the manipulation was performed from day 8 to day 12 (Figure 2.3 D), and hydathode number and the length: width ratio of the lamina increased more gradually in defoliated plants than in controls (Figure 2.3 E, F). These results indicated that defoliation delays phase change.

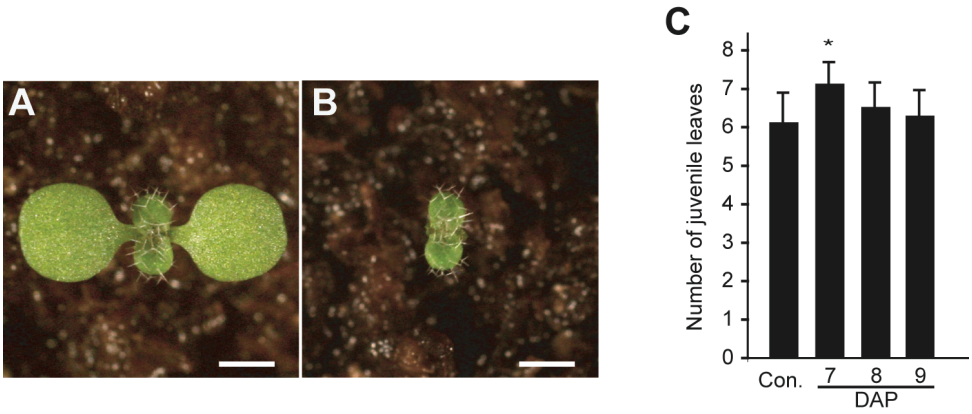


Figure 2.2: Cotyledon ablation does not affect phase change.

A) and B) 8-day old seedling before (A) and after (B) cotyledon ablation. (Scale bar=2mm). C) the effect of cotyledon ablation on the appearance of abaxial trichome. Cotyledon ablation performed at day 7 delays the onset of abaxial trichome. However, the growth of treated plants is severely retarded. No effect on trichome distribution was observed when the ablation was done at day 8 and day 9.

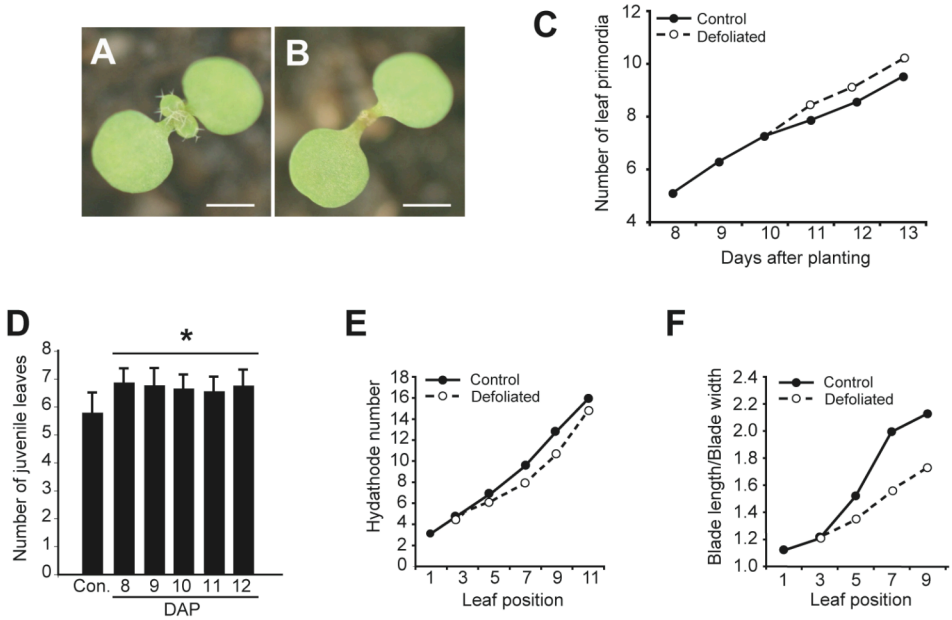


Figure 2.3: Defoliation delays phase change.

A) and B) 8-day old seedling before and after defoliation. (Scale bar = 2 mm). C) Defoliation accelerates leaf initiation. D) Defoliated plants produce more leaves lacking abaxial trichomes (juvenile leaves) than the wounded control (Students T-test $P<0.05$, n = 20, error bars = s.d.). E) The number of hydathodes (n=10) and F) the length:width ratio of successive leaves (n=7) in wounded control and defoliated plants.

The number of leaf primordia present at the time of cotyledon or leaf ablation was determined by dissecting a matched set of plants expressing LFY::GUS, a reporter that is expressed young leaf primordia. This analysis revealed that the first transition leaf (leaf 5 or 6) was produced 7 or 8 days after germination, and was therefore present on many, if not most of the seedlings used for leaf ablation (Figure 2.3 C). The fact that leaf ablation was capable of changing the morphology of this pre-existing leaf indicates that the loss of leaf primordia has a rapid effect on the identity of the shoot apex, and implies that leaves 1 and 2 begin produce a phase change signal before, or shortly after, the stage at which they were ablated, i.e., at a length of about 1 mm.

We tested the hypothesis that defoliation acts by affecting the expression or activity of miR156 by examining the effect of this treatment on two mutants, *sqn-1* and *ago1-45*, that have reduced miR156 activity (Smith et al., 2009). Leaf ablation had no effect on the timing of abaxial trichome production in both mutants (Figure 2.4 A), indicating that miR156 is required for the effect of leaf ablation on vegetative phase change. We then examined the effect of leaf ablation on the expression of miR156 and three genes repressed by miR156: miR172, *SPL3* and *SPL9*. miR156 is present at high levels early in shoot development and declines during vegetative phase change, whereas miR172, *SPL3* and *SPL9* have the opposite expression pattern (Aukerman and Sakai, 2003; Wu and Poethig, 2006; Jung et al., 2007; Wang et al., 2009b; Wu et al., 2009). Consistent with their juvenilized phenotype, defoliated plants had higher levels of miR156 and lower levels of miR172 than wounded controls (Figure 2.4 B). To examine the effect of defoliation on the expression of *SPL3* and *SPL9* we took advantage of

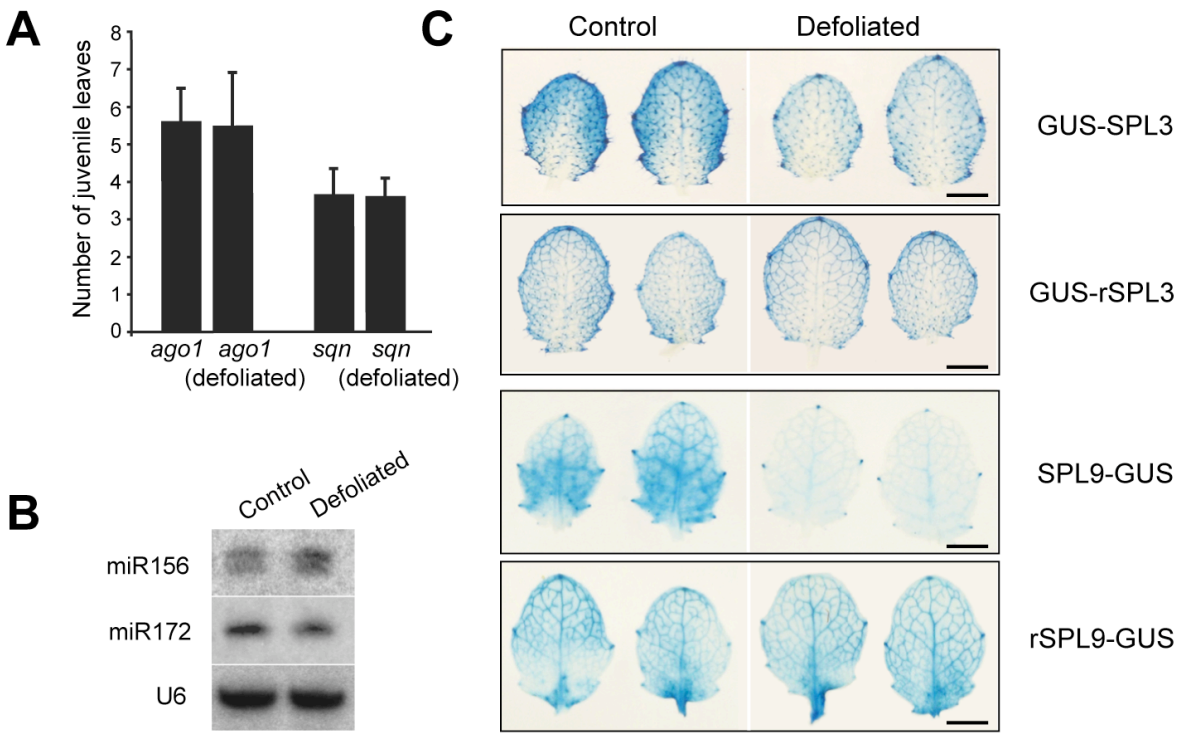


Figure 2.4: The effect of defoliation on vegetative phase change is mediated by miR156.

A) Defoliation has no effect on the number of leaves without abaxial trichomes in *ago1-45* and *sqn-1* mutants (n = 13; ± s.d; P>0.05.). B) RNA blots of 17-day-old plants; miR156 is elevated and miR172 is reduced in defoliated plants compared to wounded controls. U6 was used as a loading control. C) GUS activity in the 5th leaf primordium of transgenic plants expressing miR156-sensitive (GUS-SPL3, SPL9-GUS) and miR156-resistant (GUS-rSPL3, rSPL9-GUS) reporters for SPL3 and SPL9. Defoliation reduces the expression of the miR156-sensitive reporter, but not the miR156-resistant reporter. Scale bar = 1 mm.

reporter lines containing miR156-sensitive or miR156-resistant genomic constructs of these genes fused to GUS. Leaf ablation decreased GUS expression in the fifth leaf primordia of plants expressing the miR156-sensitive transgenes, but had no effect on the expression of the miR156-insensitive reporters (Figure 2.4 C). This result demonstrates that the decrease in the expression of the miR156-sensitive reporters in defoliated plants is mediated by miR156, which is consistent with the increased level of miR156 in these plants (Figure 2.4 B).

miR156 is encoded by 8 genes in Arabidopsis. We used quantitative RT-PCR to measure the abundance of the primary transcripts of four of these genes (*MIR156A*, *MIR156B*, *MIR156C* and *MIR156H*) to determine if the increase in miR156 in defoliated plants is mediated at a transcriptional or post-transcriptional level. Defoliation increased the expression of the primary transcripts of *MIR156A* and *MIR156C* approximately 2-fold but had no effect on the expression of *MIR156B* and *MIR156H* (Figure 2.5), suggesting that only some *MIR156* genes respond to defoliation. This result also indicates that defoliation acts by increasing the transcription of *MIR156* genes, rather than by increasing the rate of miRNA processing. If defoliation increased the level of mature miR156 transcripts by enhancing the processing of the primary transcripts it would be expected to produce a decrease—not an increase—in the abundance of the primary transcripts.

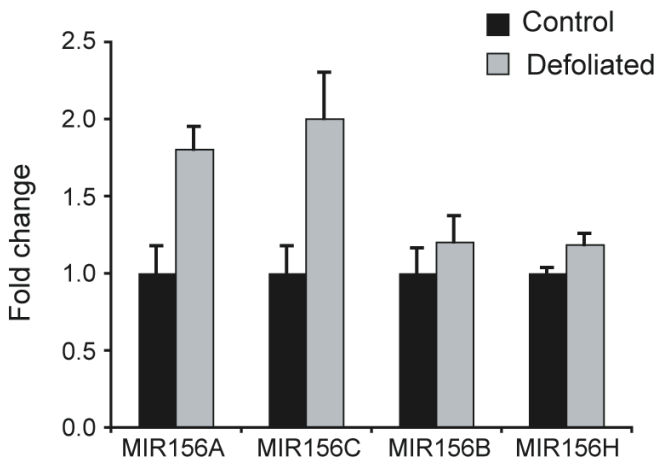


Figure 2.5: Quantitative RT-PCR of the primary transcripts of *MIR156* genes in control and defoliated plants (n = 3, ± s.d.). Samples were normalized using *ACTIN*.

2.3.3. Defoliation induces miR156 expression in other species

In *N. benthamiana*, miR156 normally decreases between 10 and 15 days after planting (Figure 2.6 A). Removal of one or two cotyledons from 14-day-old plants had no effect on the expression of miR156, but removal of a single leaf primordium produced a significant increase in the level of this miRNA (Figure 2.6 B). Culturing shoot apices from adult maize plants in vitro causes either complete (Irish and Karlen, 1998) or partial (Orkwiszewski and Poethig, 2000) rejuvenation of the shoot apex, depending on the number of leaf primordia remaining on the explant. An analysis of the effect of this treatment on the expression of miR156 and miR172 revealed that miR156 begins to increase within 1 day after explanting shoot apices into culture, and is elevated quite significantly in 6-day-old explants (Figure 2.6 C). miR172 decreased in a complementary fashion (Figure 2.6 C). *Acacia Mangium* was one of the first heteroblastic species described by Goebel (Goebel, 1900). It produces pinnate leaves in the juvenile phase and phyllodes in the adult phase. Such heteroblastic change is associated with the change of miR156 level (Wang et al., 2011). Removing the first two leaves in *Acacia Mangium* resulted in a similar increase of miR156 (Figure 2.6 D). As a consequence, defoliated plants generated more pinnate leaves compare to control (Figure 2.6 E). Thus, defoliation has the same effect on miR156 expression in herbaceous and woody plants.

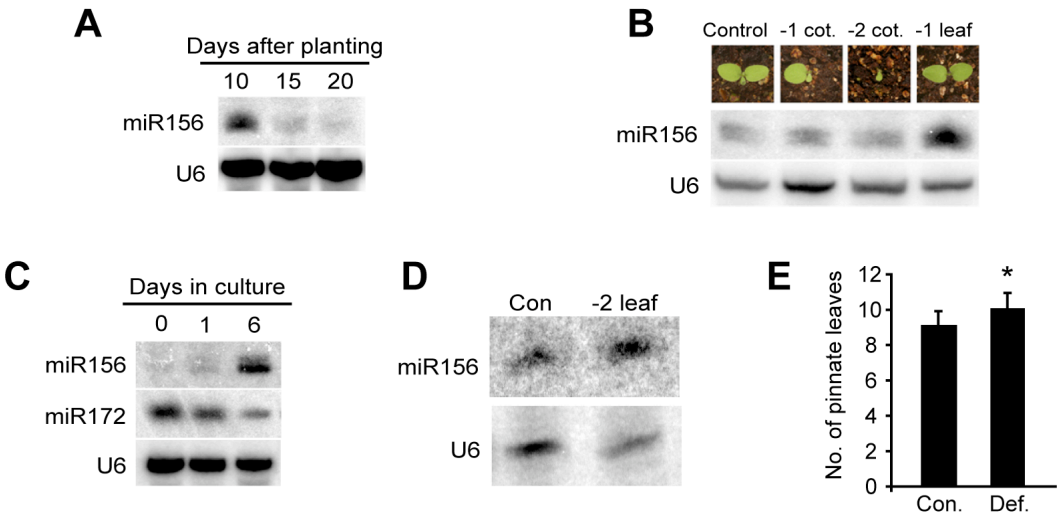


Figure 2.6: The effect of defoliation on miR156 levels in maize, *N. benthamiana* and *Acacia Mangium*

A) miR156 expression decreases between 10 and 15 days after planting in *N. benthamiana* seedlings. B) The expression of miR156 in *N. benthamiana* seedlings is unaffected by cotyledon ablation, but increases in response to defoliation. -1 cot.: one cotyledon ablated; -2 cot.: two cotyledons ablated; -1 leaf: first leaf ablated. C) miR156 expression increases and miR172 expression decreases in shoot apices of adult maize plants growing in culture. D) Defoliation induces miR156 in *Acacia* shoot. E) Defoliation increases the total number of pinnate juvenile leaves. (*: $P<0.01$) U6 was used as a loading control.

2.4.Discussion

Studies of the source and chemical identity of the signals that regulate vegetative phase change have been complicated by the large number of species and the wide variety of traits that have been employed in these studies. With few exceptions, these studies have focused on a single trait, for example, leaf shape or adventitious root production. Although this trait may mark a more general change in the character of the shoot, in most cases this has not been demonstrated. This is a serious issue because many different factors affect the expression of heteroblastic traits during shoot development, and it can be difficult to distinguish traits that vary as a consequence of vegetative phase change from those that vary as a result of floral induction or some other change in the physiology of the shoot (Lee and Richards, 1991; Jones, 1999). For example, while leaf shape is a good marker of phase identity (Goebel, 1900), changes in light intensity can affect leaf shape in ways resemble the effect of vegetative phase change (Njoku, 1956a), without necessarily operating by the same mechanism (Jones, 1995). This has made it difficult to decide if previous studies of the effect of defoliation on leaf development (Ashby, 1948; Njoku, 1956b), are relevant to the mechanism of vegetative phase change.

Our analysis of the effect of root and leaf ablation on the expression of phase-specific morphological traits and the expression of miR156 suggests that the timing of vegetative phase change is regulated by factors produced by leaves or leaf primordia, not the root system. Specifically, we found that complete ablation of the root system from very early in embryo development did not significant affect the timing of abaxial production and in most cases did not have a major effect on shoot morphology. In

contrast, removal of the first two leaves produced a modest, but significant delay in the onset of abaxial trichome production and produced long lasting effects on both hydathode number and leaf shape that are consistent with a juvenilized phenotype. The observation that defoliation affects the morphology of all of the leaves on the rosette, rather than only a few, is the expected result if this treatment acts by delaying the onset of vegetative phase change. An alternative possibility is that defoliation transiently affects shoot development by modifying the morphology of leaves produced shortly after this treatment. If this were the case, we would have to conclude that this treatment affects events downstream of vegetative phase change, not the timing of the process itself. Conclusive evidence that leaves are the source of a phase-change signal was provided by the observation that defoliation increases the expression of miR156 and decreases the expression of its targets, as well as by the observation that mutations that interfere with the activity of miR156 block the effect of defoliation on abaxial trichome production.

What is the nature of this leaf-derived signal? Gibberellin promotes the expression of adult vegetative traits in both Arabidopsis and maize (Evans and Poethig, 1995; Chien and Sussex, 1996a; Telfer et al., 1997) and has also been shown to affect phase change in woody plants (Zimmerman et al., 1985). But, while it is conceivable that the loss of leaf-derived GA accounts for the effect of defoliation on vegetative phase change, Wang and colleagues (Wang et al., 2009b) have shown that GA has no effect on the expression of miR156 in Arabidopsis. Given that leaf ablation elevates miR156 expression, we think it is unlikely that GA is responsible for the effect of leaves on vegetative phase change. IAA and cytokinin also have no effect on miR156 expression (Wang et al., 2009b), excluding a role for these hormones as well. A more likely

possibility is carbohydrates. Numerous studies have shown that light intensity has significant effects on heteroblastic features of leaf morphology, and the extensive studies of Allsopp and other early investigators suggested that this effect is largely attributable to carbohydrate supply (reviewed in (Allsopp, 1965; Allsopp, 1967b)). In general, in low light condition or with low levels of exogenous sugar, plants produce leaves with juvenile morphology, whereas high light, or high sugar levels, promotes adult leaf type. Allsopp (Allsopp, 1954) believed that carbohydrates exerted their effect by modifying the size of the shoot apical meristem, but experiments on excised leaf primordia suggest that sugar controls leaf development more directly (Sussex, 1960). Whether carbohydrates play a regulatory role in vegetative phase change, or act specifically to regulate leaf morphogenesis, remains to be determined.

3. THE HXK1-DEPENDENT GLUCOSE SIGNALING REPRESSES MIR156

3.1. Abstract

The timing of developmental transition is largely influenced by the availability of various nutrients. In plants, the biosynthesis, metabolism and allocation of different sugars is vital for development. During the process of shoot maturation, sugar availability affects the onset or progress of many traits that are differentially expressed in the juvenile and adult phases. The question remains whether sugar determines the timing of phase change and what the underlying molecular mechanism is. In this study, we found that supply of glucose/sucrose in growth medium accelerated phase change in Arabidopsis, which is associated with a reduction of miR156 abundance. On the contrary, miR156 is highly accumulated in *ch1*, a mutant with reduced photosynthesis, resulting in a prolonged juvenile phase. Blocking the function of miR156 is sufficient to suppress the delayed phase change in *ch1*. Analysis of *MIR156* precursors and the expression of promoter reporters showed that sugars repressed the transcription of some *MIR156* loci, thus leading to a decrease of the mature miR156 level and, consequently, an increase of *SPL* genes. Such sugar-mediated repression of miR156 depended on the signaling role of HXK1, which is a primary glucose sensor in Arabidopsis. These data indicated that sugar promotes phase change by repressing the expression of *MIR156* genes.

3.2. Background

Plants sense nutrients, such as sugars, to coordinate development, growth, and responses to abiotic or biotic stresses (Coruzzi and Zhou, 2001; Gibson, 2005). Sugars regulate many developmental processes, ranging from seed germination to leaf morphogenesis to pollen formation (Gibson, 2005).

Sugar availability also influences the progress of heteroblastic development, a major readout of vegetative phase change. In general, glucose, fructose, and sucrose promote the adult leaf form. For example, in *Marsilea drummondii*, supplying glucose, sucrose, or fructose in medium increased the leaf segmentation, a feature characteristic of adult leaf form (Allsopp, 1955). On the contrary, when well-developed sporelings with segmented leaves were grown in a sugar-depleted medium, reversion to a simpler form occurred after several months (Allsopp, 1955). Similar effects of sugars on heteroblastic development were observed in *pteridophytes* (Wetmore et al. 1953,1954) and in *Ipomoea purpurea* (Njoku, 1971). One explanation for the effect of sugar on leaf form is that sugar may alter leaf shape by promoting shoot maturation. An alternative interpretation is that sugar can regulate leaf morphogenesis independently of shoot maturation. Sussex (1960) observed that the number of pinnule pairs on excised leaf primordia of *Osmunda cinnamomea* increased when the concentration of sucrose in medium rose (Sussex, 1960), suggesting that sucrose regulates leaf morphogenesis after leaf initiation. Although it is not clear how sugar modify heteroblastic traits, these two possibilities are not mutually exclusive. *ATHB13* provides a potential molecular link between sugar concentration and leaf shape. Constitutive expression of *ATHB13* inhibited lateral cell expansion, resulting

in narrow cotyledons and leaves. Such phenotype was only evident when sugar was added to the growth medium, so ATHB13 is part of a sugar-signaling pathway that regulates leaf shape by modifying cell expansion (Hanson et al., 2001).

In addition to leaf shape, sugars also regulate other phase-related traits, such as floral induction, epidermal patterning, internode length and rooting ability. An increase of sugar level in leaf exudates is associated with floral induction (Bodson and Outlaw, 1985; Corbesier et al., 2002; Lejeune et al., 1991; Lejeune et al., 1993; Milyaeva and Komarova, 1996; Milyaeva et al., 1996; Perilleux and Bernier, 1997; Roldan et al., 1999; Wong et al., 2009). Arabidopsis plants grown on 5% sucrose had significantly more adult leaves than normal plants, suggesting that a high concentration of sucrose prolonged the adult vegetative phase (Ohto et al., 2001). In tobacco, reducing photosynthesis by knocking down the RUBISCO small subunit (RBCS) specifically delayed shoot maturation in terms of leaf shape and internode distance (Tsai et al., 1997). A high rooting ability is usually considered as a juvenile feature. Fifteen to sixty mM of exogenous sucrose promoted the formation of adventitious roots on Arabidopsis hypocotyle, while increasing the concentration to 150mM resulted in an inhibitory effect (Takahashi, 2003). These observations implicate that sugar regulates various traits associated with phase change.

Although the link between sugar availability and phase change is clear, studies of the underlying molecular mechanism are hindered by the pleiotropic roles of sugars in plant development. Sugars are an energy source, and are the building blocks of macromolecules such as cellulose, DNA, and RNA. Sugars also regulate osmotic pressure and protein modifications. In addition, some sugars serve as signaling molecules

to directly initiate a signaling cascade (Koch, 2004). Changes in the sugar level may alter the global energy state as well as the specific developmental pathways. Most of the assays designed to examine the effect of sugars on a certain development process, such as phase change, were done by applying sugars exogenously. Although the identity of the sugars added to the medium is known, the actual molecules being sensed by the plant is unknown. Sucrose, glucose, and fructose are inter-convertible in plant cells. Recent evidence showed that trehalose-6-phosphate controls some sugar responses, suggesting that not only sugar itself but also sugar derivatives may trigger the signaling response (van Dijken et al., 2004; Gomez et al., 2005; Gomez et al., 2006; Chary et al., 2008; Gomez et al., 2010). Furthermore, some studies suggest that plants are not only measuring the absolute sugar concentration but also a relative C:N ratio. For example, the repressive role of a certain concentration of glucose on photosynthetic gene expression is more evident in a nitrate-deprived medium (Moore et al., 2003). The existence of multiple sugar sensors, metabolic enzymes, and transporters involved in sugar metabolism and allocation further complicates this issue. Three enzymes involved in carbohydrate metabolism – ADPglucose pyrophosphorylase, sucrose synthase, and an SNF1-like kinase – are expressed in an asymmetric pattern within the meristem (Pien et al., 2001). In addition, differential distribution of invertase in the cytosol or apoplast caused either a late-flowering or early-flowering phenotype, suggesting that a sophisticated regulation of sugar type and allocation exists in meristem (Heyer et al., 2004).

Within the sugar-signaling network, hexokinase (HXK1) is a primary sensor of glucose (Jang et al., 1997). In Arabidopsis, a mutant of HXK1, *gin2-1* (*glucose*

insensitive 2), is insensitive to the inhibitory effect of high glucose concentration. In addition to the glucose hyposensitivity, *gin2-1* exhibits pleiotropic defects, such as a smaller leaf size, a delayed leaf senescence, and a reduced cell expansion. The developmental role of HXK1 is partly executed through its widespread interactions with plant hormone pathways. Glucose signaling interacts with ABA, auxin, cytokinin, and the ethylene signaling pathways. For example, like *gin2*, the constitutive ethylene biosynthesis mutant (e.g. *eto1*) is insensitive to the glucose (Moore et al., 2003). In Arabidopsis, HXK1 has dual roles in glucose homeostasis. The enzymatic role of HXK1 is to catalyze glucose to glucose-6-phosphotase; its signaling role is to serves as a glucose sensor and directly regulate gene transcription. These two roles are not mutually dependent. Moore et al. (2003) found that glucose phosphorylation – which is a major output of the catalytic function of – was not correlated with the quantitative indicators of glucose signaling such as the chlorophyll level and the photosynthetic gene expression. Furthermore, mutated forms of HXK1 with point mutations at the catalytic site (S177A and G104D) only restored the signaling function of HXK1 (e.g. repressing CAB (chlorophyll a/b binding protein) genes and inhibiting seedling development), but not its glucose phosphorylation capacity. These data suggested that HXK1 may regulate plant growth and development independent of its catalytic function (Moore et al., 2003). This function probably executed by an HXK1-containing nuclear complex, which interacts with the vacuolar H^+-ATPase B1 (VHA-B1) and the 19S regulatory particle of proteasome subunit (RPT5B). This complex can directly bind to the promoter of *CAB*, to repress its transcription (Cho et al., 2006).

Due to the complexity of sugar-involved processes, little is known about the underlying molecular mechanism how sugar regulates phase change. Several important questions remain: (i) Does sugar regulate the timing of phase change or the specification of downstream phenotypes? (ii) Does sugar regulate phase change through miR156? (iii) What signaling pathway is involved in the sugar-mediated regulation of phase change? In this study, we investigated the role of glucose in regulating vegetative phase change in Arabidopsis. An exogenous supply of sucrose/glucose repressed miR156 expression and induced the early appearance of adult traits. On the other hand, reducing the endogenous sugar level using a *ch1* mutation significantly delayed phase change in short-day condition. This delay was completely rescued by blocking miR156 function. The sugar-induced effect was due to its repressive role on the transcription of some *MIR156* genes. Furthermore, HXK1 is required for sugar-mediated repression of miR156, and the catalytic function of HXK1 is dispensable for regulating miR156 at an early stage. In conclusion, our results provided a molecular link between glucose signaling and the miR156-regulated developmental transition.

3.3.Results

3.3.1. Glucose suppresses defoliation-induced increase of miR156

The results in Chapter II demonstrate that a leaf-derived signal promotes phase change by repressing miR156 expression. To identify the nature of this signal, different substances were mixed with agarose gel and applied onto the wound surface generated in defoliation (Figure 3.1 A). The candidate signaling substance is expected to rescue the high level of miR156 induced by defoliation. This assay was first tested using leaf extract as a positive control. Applying the leaf extract processed from 1 cm *N. benthamiana* leaf primordia significantly suppressed the elevated level of miR156 after defoliation (Figure 3.1 B). Since leaves are a major source of photosynthetic products, we tested the ability of glucose to repress miR156. Similar to leaf extract, 300mM of glucose rescued the defoliation-induced increase of miR156 (Figure 3.1 B). However, neither glucose nor the leaf extract returned the expression of miR156 to the control level. This may be due to the limited delivery efficiency of the rescue assay, or the loss of other signaling substances that are sensitive to manipulation, such as RNA. Whatever the case, this assay suggests a role for sugar in regulating the expression of miR156.

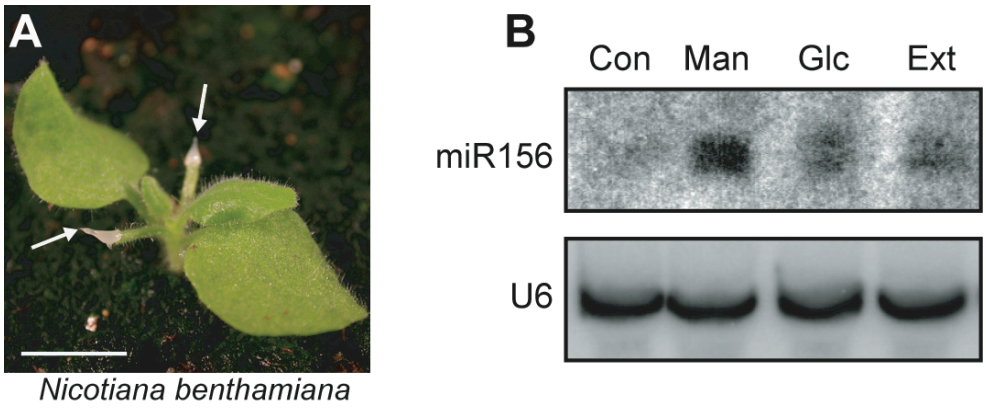


Figure 3.1: Exogenously supplied glucose rescues the elevated level of miR156 after defoliation.

A) A three-week old *N. benthamiana* with agarose gel on ablated leaf petioles. Different substances are added in agarose gel to test their effects on miR156 level. Scale bar=1cm. B) Applying glucose and the leaf extract suppresses the elevated miR156 level induced by defoliation. Con: untreated control; Man: mannitol; Glc: glucose; Ext: leaf extract. The mannitol and glucose concentration is 300mM.

3.3.2. exogenous sugar represses miR156 in Arabidopsis

We further examined the accumulation of miR156 in Arabidopsis seedlings germinated on plates with different sugar and sugar analogues. At a concentration of 10mM, glucose and fructose repressed the accumulation of miR156 (Figure 3.2 A). This was not due to a change in osmotic pressure because sugar analogues such as mannitol, sorbitol, and O-methylated-glucose did not repress the miR156 level (Figure 3.2 A). To exclude the possibility that the reduction of miR156 was a secondary result of a different growth rate, the 6th leaves from miR156 sensitive or insensitive SPL9 reporters were cultured in different media. The results showed that the expression of GUS from the miR156 sensitive SPL9 reporter (SPL9-GUS) increased in the glucose and fructose medium, but not the mannitol, sorbitol, or the O-methylated glucose medium (Figure 3.2 B). On the contrary, the GUS level from the miR156-insensitive SPL9 reporter (rSPL9-GUS) did not change with any of these substances, suggesting that the increase of *SPL9* expression in glucose and fructose medium was due to a reduction of the miR156 level. Furthermore, SPL9 expression increased within a relatively short time (8 hours), arguing that the reduction in miR156 was not a secondary result of a change in growth rate. All these data indicate that sugars, such as glucose and fructose, can suppress the accumulation of mature miR156 in Arabidopsis, resulting in an increase in the expression of its target genes, such as *SPL9*. Consistent with this molecular change, plants growing on sucrose deficient media generated more juvenile leaves than those on a medium supplemented with 4% sucrose (7.4 ± 0.6 vs 5.4 ± 0.8, $p<0.01$), indicating that the change in miR156 and *SPL* expression is functionally significant.

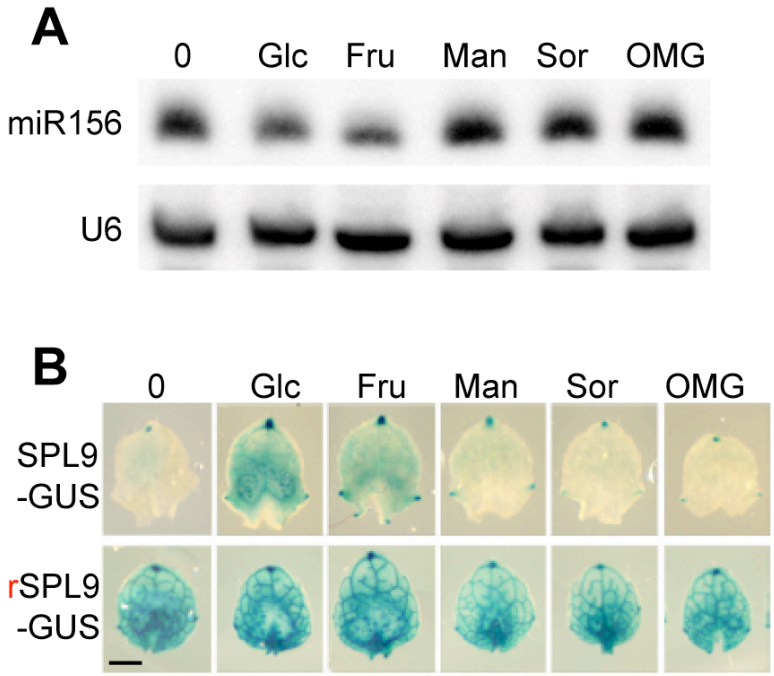


Figure 3.2: Glucose suppresses miR156 expression in Col-0.

A) The different effects of sugar analogues on miR156 expression. Glucose (Glc) and fructose (Fru), but not mannitol (Man), sorbitol (Sor) and O-methylated glucose (OMG), can suppress miR156 level when seeds are grown on plates. U6 is a loading control. B) The detached leaf culture assay shows that leaves with SPL9-GUS have more intensive GUS staining in glucose and fructose medium. The 6th leaves are detached from corresponding transgenic plants, and cultured for 8 hours in different medium. Scale bar=1mm.

3.3.3. Mutation in ch1 delays phase change

To test whether altering the endogenous sugar level affects miR56 level, we examined phase specific phenotypes in the *ch1* (*chlorina 1*) mutant. The *ch1* mutant harbors a mutation in chlorophyllide *a* oxygenase, which blocks the biosynthesis of chlorophyllide b (Espineda et al., 1999; Oster et al., 2000). The mutant appears yellow green and grows slower than the wild type Col-0 (Figure 3.3 A). Interestingly, the *ch1* mutant displayed significantly delayed phase change. In short day conditions, *ch1* mutants generated 5 more juvenile leaves than the wild type (Figure 3.3 A, B). Compared to wild-type plants, the leaves of *ch1* plants were round and with smooth margin (Figure 3.3 A, B). We did not observe a delay in abaxial trichome production when growing *ch1* in long days, although the leaves were still round. To examine whether the delayed phase change in *ch1* depended on miR156 function, we introduced *ch1* into a *35S::MIM156* background, in which miR156 function was blocked by target mimicking. Homozygous plants harboring *ch1* and *35S::MIM156* produced abaxial trichomes on the first leaf, and the leaves were elongated and serrated, which are characteristics of *35S::MIM156* plant (Figure 3.3 C, D), indicating that the delayed phase change in *ch1* was suppressed by *35S::MIM156*. The interaction between *ch1* and *35S::MIM156* implies that the delayed phase change observed in *ch1* depends on the normal function of miR156.

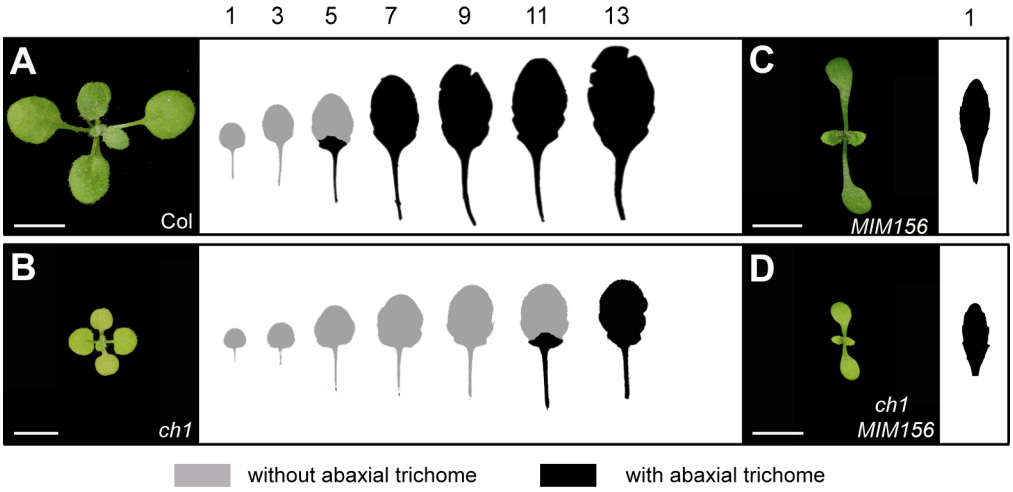


Figure 3.3: The prolonged juvenile phase in *ch1* is suppressed by *35S::MIM156*.

A) A wild type Col generates about 6 juvenile leaves (5.7±0.9, n=24) in short day condition. B) A *ch1* mutant produces significantly more juvenile leaves (10.9±0.8,n=24) than Col-0. C) and D) A *ch1 35S::MIM156* (D) plant resembles *35S::MIM156* (C) in leaf shape and early appearance of trichome. Both of them produce abaxial trichomes on leaf 1. (n=24 for *35S::MIM156*; n=24 for *ch1 35S::MIM156*). Gray color indicates abaxial leaf surface without trichomes; black color indicated abaxial leaf surface with trichomes. Numbers indicate the position of each leaf on a shoot. Scale bar=1cm.

The change of miR156 and the *SPL* genes in *ch1* supports the conclusion drawn from genetic data. Northern blots showed that more miR156 was accumulated in *ch1* than that in wild type at each time point tested (Figure 3.4 A). However, the temporal expression pattern of miR156 was unaffected by *ch1*. Consistent with the high level of miR156, the expression of the SPL3-GUS reporter was reduced in a *ch1* background (Figure 3.4 B, C). In wild-type plants, pSPL3::SPL3-GUS expression was very low in cotyledons and the first two leaves, and became evident in leaf 3 and leaf 4 (Figure 3.4 B). In *ch1*, the reporter showed a similar expression pattern, but with significantly reduced staining intensity (Figure 3.4 C). The transcript level of *SPL3* as well as *SPL9* and *SPL13* also dropped in *ch1* (Figure 3.4 D). The change in miR156 and *SPL* expressions in *ch1* could be due to multiple physiological and developmental defects caused by reduced chlorophyllide b. To explore this possibility, I tested the effect of glucose on miR156 expression in *ch1*. *ch1* seedlings grown on 50mM of glucose had significantly reduced level of miR156 (Figure 3.4 E). Increasing the glucose concentration to 100mM did not further reduce miR156 expression, suggesting that a threshold exists for glucose uptake or glucose response (Figure 3.4 E). Taken together, these data demonstrate that *ch1* has elevated expression of miR156, which may be due to a reduction in sugar content.

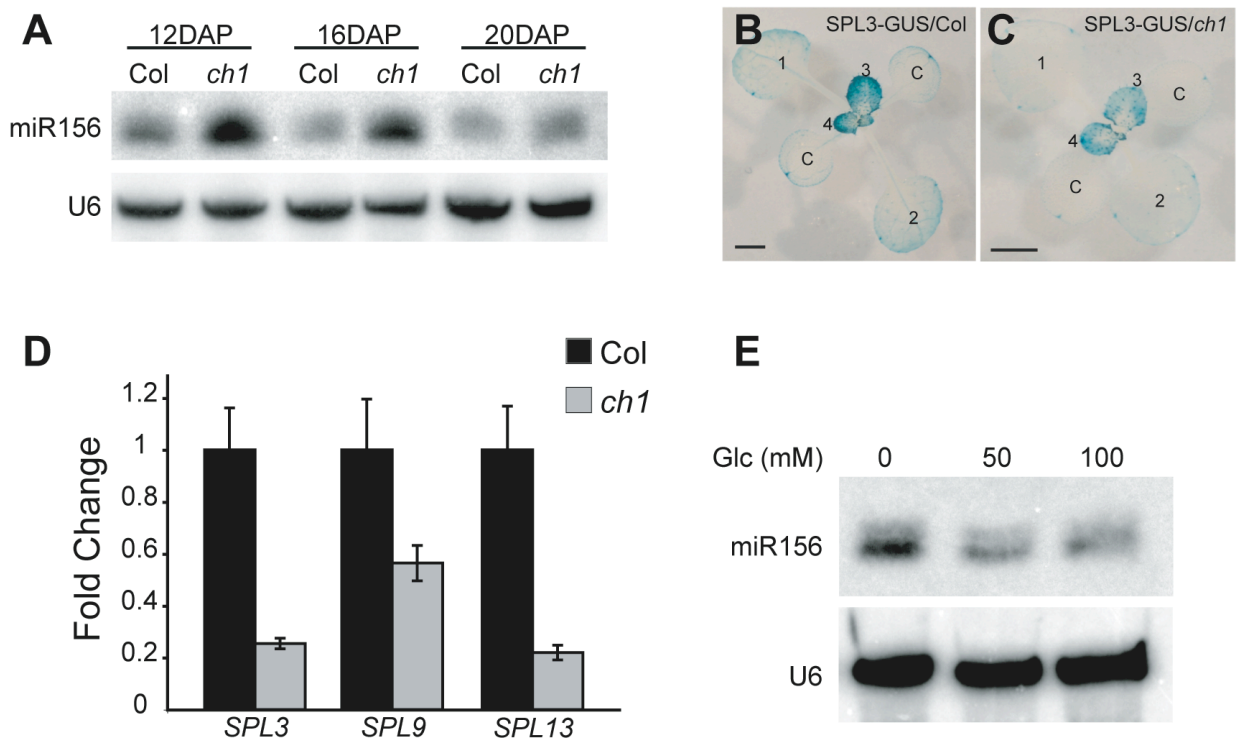


Figure 3.4: Expression of miR156 and *SPL* genes in *ch1*.

A) miR156 is over-accumulated in *ch1*. At each sampled time point, *ch1* accumulated more mature miR156 than Col, although the temporal expression pattern remained. DAP stands for days after planting. U6 is a loading control. B) And C) SPL3-GUS+ is expressed at a low level in *ch1* (C), comparing with it in Col-0 (B). To match the developmental stage of wild type, *ch1* plant was harvested 5 days later. D) The transcripts level of *SPL3*, *SPL9* and *SPL13* are decreased in *ch1* mutant. The reduction of *SPL3* and *SPL13* is more significant than that of *SPL9*. E) The northern was performed in *ch1* background. The high level of miR156 in *ch1* can be suppressed by glucose. 50mM of glucose was sufficient to suppress the level miR156, while 100mM of glucose did not generate further reduction. U6 is a loading control. Scale bar in (B) and (C)=2mm.

3.3.4. Sugar suppress the transcription of MIR156 genes

To address whether the sugar-mediated regulation of miR156 is at a transcriptional or posttranscriptional level, the expression of *MIR156* precursors in response to sugar was analyzed. To obtain a strong signal for real-time PCR, the *se* mutant was used instead of the wild type. The processing of primary miRNAs is inefficient in *se*, leading to an accumulation of miRNA precursors. *MIR156A*, *C*, *F*, and *H* were down regulated by exogenous glucose, while *MIR156B* and *D* did not change (Figure 3.5 A). We could not get reproducible results for *MIR156E* and *MIR156G*, probably due to their very low expression level. Deep sequencing of small RNAs and quantitative real-time PCR (Willmann, Koo unpublished) demonstrates that *MIR156A* is temporally expressed and makes a significant contribution to the mature miR156 pool, so we generated a GUS reporter line for *MIR156A* and examined its response to sugar (Figure 3.5 B). For this purpose, we took a 7kb genomic sequence spanning the *MIR156A* locus and replaced the stem loop structure of *MIR156A* with GUS+. The expression of this reporter was repressed by 4% sucrose or 1% glucose, which is consistent with the real-time PCR result. I subsequently tested the sugar response of 6 truncated versions of *MIR156A* reporter (Figure 3.5 B). Because the transcription start site is not well characterized, we define the first nucleotide of mature miR156 as +1. Two truncated reporters, P156A (-1317) and P156A (+2530), maintained their sugar response, although deleting 1.2kb at the 3' end (P156A(+2530)) reduced its transcriptional activity. The other two truncations, P156A(-482) and P156A(+929), completely abolished the GUS expression, indicating that the regions from -1317 to -482 and from +929 to +1447 are

required for transcriptional activity. Interestingly, plants carrying P156A(+1447) or P156A(+1934) expressed the same level of GUS, albeit weak, in 0% and 4% of sucrose, suggesting that the sugar response element is located in a 500bp region between +1934 and +2530. These data support that sugar mediates the transcription activity of some *MIR156* loci.

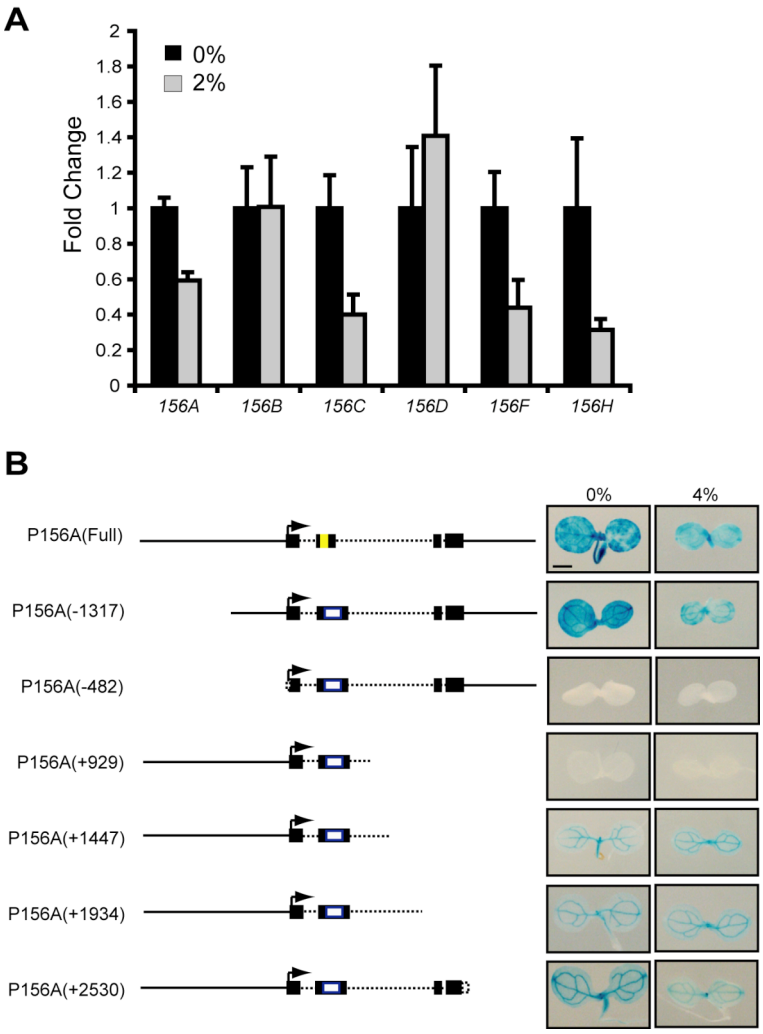


Figure 3.5: Transcriptional regulation of *MIR156* precusors

A) A subgroup of MIR156 precursors responds to glucose. *MIR156A*, *C*, *F*, *H* are down regulated with a 50mM glucose supply in medium, *MIR156B* and *D* were not changed. The RNAs are extracted from *se* mutant grown on 0mM or 50mM glucose medium. B) Promoter truncation analysis for *MIR156A* genomic region. The full length of *MIR156A* reporter, P156A(Full), is repressed by 4% of sucrose. One 5' truncation and one 3' truncation, P156A(-1317) and P156A(+2530), do not alter sugar response. P156A(+929) and P156A(-482) depleted transcriptional activity. Two truncations, P156A(+1447) and P156A(+1934) had reduced transcription level and lost sugar response. Each staining figure was representative of two independent transgenic lines. Black rectangle=exon; yellow rectangle=mature miR156; white rectangle=GUS+; solid line=intergenic sequence; dashed line=intron; arrow=putative transcriptional start site. Bar=2mm.

3.3.5. HXK1 is required for sugar-mediated repression of miR156

Previous work showed that the glucose signaling regulates gene expression via an HXK1-dependent manner (Moore et al., 2003; Cho et al., 2006). The mutant of *HXK1*, *gin2-1*, is morphologically similar to wild type L*er*, albeit grows slightly slowly (Figure 3.6 A, B). However, *gin2-1* accumulated a low level of miR156 in a sugar starvation condition (Figure 3.6 E). Interestingly, the expression of miR156 was no longer suppressed by 10mM of glucose in the *gin2-1* background, indicating that the HXK1 function is required for the sugar-mediated regulation of miR156 (Figure 3.6 E). HXK1 executes two independent roles: an enzymatic role and a signaling role (Moore et al., 2003). To address which of them is required in repressing miR156, the miR156 level was examined in the *S177A/gin2-1* background. S177A is a mutated form of HXK1, which is catalytically inactive but reserves its signaling role in repressing photosynthesis genes. Introducing the S177A into the *gin2-1* background under a constitutive 35S promoter restored the HXK1 mediated signaling role, but not its catalytic function (Moore et al., 2003). The *gin2-1* mutant carrying the catalytically inactive HXK1 (*S177A/gin2-1*) or the intact HXK1 (*HXK1/gin2-1*) are morphologically similar to each other (Figure 3.6 C, D). Surprisingly, *S177A/gin2-1* still reposed to sugar in repressing miR156 as L*er* and *HXK1/gin2-1* (a complete rescue) (Figure 3.6 F). This result suggests that the enzymatic function of HXK1 is dispensable for repressing miR156. The phenotypic analysis showed that the reduced level of miR156 in *gin2-1* was functionally significant. Grown in washed soil, *gin2-1* plants produced fewer juvenile leaves (Figure 3.6 G). The difference was small but statistically significant. More importantly, introducing S177A into *gin2-1*

restored the trichome phenotype, suggesting that the morphological defect was due to the deficient signaling pathway (Figure 3.6 G). In conclusion, HXK1 is essential in regulating the miR156 level, and its signaling role conducts a crosstalk between the sugar state and the miR156 level.

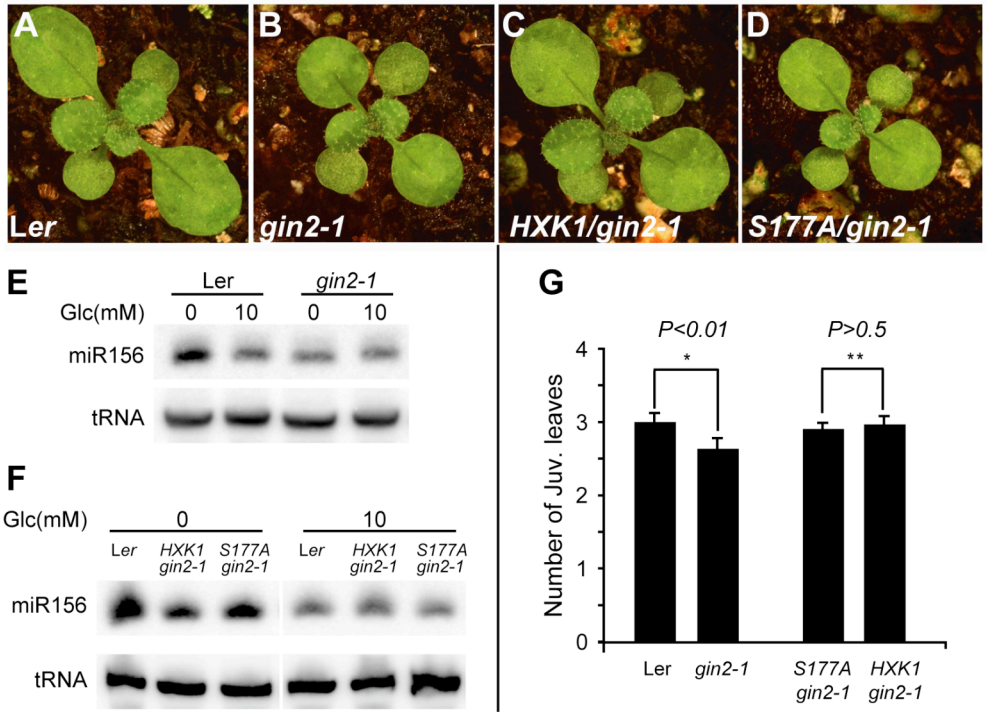


Figure 3.6: The signaling role of HXK1 is required for glucose-mediated repression of miR156.

A)-D) a 14-day old L*er* (A), *gin2-1* (B), *S177A*/*gin2-1* (C) and *HXK1*/*gin2-1* (D). E) *gin2-1* has low level of miR156 compared with wild type, and it blocks the glucose mediated repression of miR156. F) *S177A*/*gin2-1* with defective enzymatic function remains the role to repress miR156 upon sugar supply. G) Trichome distribution in Ler, *gin2-1* and *S177A*/*gin2-1*. *gin2-1* had reduced number of juvenile leaves; restoring the signaling role of HXK1 in *S177A*/*gin2-1* rescues the early phase change in *gin2-1*.

3.4.Discussion

How plants link nutrition and developmental timing is an intriguing question. The decision of plants to switch between developmental programs is associated with the availability of various nutrients and their relative ratio as well as environmental factors. In this study, we discovered that sugars such as glucose and sucrose repressed the expression of miR156, thus accelerating the transition from the juvenile phase to the adult phase. On the other hand, sugar-starvation caused by *ch1* mutation resulted in a high miR156 level and a delayed phase change. The discovery that sugar affects miR156, a key regulator of developmental timing, provides a molecular link between carbohydrate availability and the regulation of phase change. These sugar responses imply that the decision to enter the next developmental phase is partially dependent on the available sugar level. Once plants sense enough sugar, which could be a sign of sufficient storage or vigor, the adult phase will be initiated by a sugar-mediated repression of miR156. Such repressive role of sugar on miR156 is consistent with the previous observation that sugar promoted leaf complexity and flowering (Sussex, 1960; Allsopp, 1967a; Roldan et al., 1999; Ohto et al., 2001), both of which are adult characters. In addition to direct sugar supply, some environmental conditions also affect phase change. For example, a high light intensity accelerated the transition from simple juvenile leaves to lobed adult leaves in *Ipomoea caerulea* (Njoku, 1956). A plausible explanation is that a high light intensity stimulates photosynthesis, thus elevating endogenous sugar level. It is interesting to note that the *MIR156* loci that responds to leaf ablation (*MIR156A* and *MIR156C*, see Chapter

II) overlap with the ones repressed by glucose, implying that the defoliation induced delay of phase change may be due to a reduction of the carbohydrate supply in shoot.

Our results indicate that the signaling role of HXK1 is important for repressing the transcription of a subset of *MIR156* loci. The HXKs are conserved glucose sensors in yeast and plants (Rolland et al., 2002). The requirement for HXK1 in sugar-mediated repression of miR156 suggests that glucose is the type of sugar that is sensed by plants. Of course, other sugars or sugar derivatives can simultaneously act in parallel pathways, which may explain the mild phase change phenotype in a *gin2-1* mutant. The weak phenotype may also be due to the redundancy between the two hexokinases and four hexokinase-like genes in Arabidopsis genome (Rolland et al., 2006). HXK1 participates in a nuclear complex and to directly regulate the gene expression (Cho et al., 2006). One possibility is that HXK1 can directly bind to the promoter of the *MIR156* genes, thus promoting their expression. Because HXK1 does not seem to have the transcriptional activity, other transcriptional factors should be involved in the sugar-dependent repression of miR156. On the other hand, sugar singling has extensive crosstalk with plant hormone signaling. For example, GA inhibits the sucrose-induced synthesis of anthocyanin by repressing dihydroflavonol reductase (DFR) (Loreti, 2008). Mutants of *SPINDLY* (*SPY*) and *RGA-like 2* (*RGL2*), two negative regulators of the GA pathway, are more resistant to the glucose-induced repression of seed germination (Yuan, 2006). During phase change, GA treatment induces the precocious appearance of the abaxial trichome, elongated leaf shape, and early flowering in Arabidopsis, which mimics the miR156 loss-of-function phenotype (Chien and Sussex, 1996b; Telfer et al., 1997; Wu et al., 2009). However, exogenous applications of 100uM GA do not change the miR156

level, indicating that miR156 is acting upstream or in a parallel pathway as GA (Wang et al., 2009a). Since the sugar signal is upstream of miR156, it seems unlikely that sugar regulates miR156 through the GA function.

The Arabidopsis genome contains 8 *MIR156* loci. Six of them (*MIR156A* to *MIR156F*) can produce mature miR156 with the identical sequence; *MIR156G* and *MIR156H* generate mature miR156 with one or two nucleotide differences, respectively. Furthermore, multiple *MIR156* loci exist in plant genomes that range from moss to monocots to dicots (Axtell, 2007). Very little is known about how the transcription of different *MIR156* loci is regulated and whether these loci play divergent roles. Xing et al. (2010) reported a differentiated expression pattern from 8 MIR156 loci during anther development. *MIR156E* and *MIR156G* reporters were barely detectable, while *MIR156A*, *H,* were strongly expressed in anthers, indicating a distinct expression pattern (Xing et al., 2010). The results presented in this work clearly demonstrate that different MIR156 loci have distinct responses to the sugar level. *MIR156A*, *C*, *F*, and *H* were repressed upon glucose supply, but not *MIR156B* and *MIR156D*. The real-time PCR signal for *MIR156E* and *MIR156G* were weak and unstable, which is consistent with the absence of GUS activity from their promoter reporters (Xing et al., 2010). The miR156 level is induced by phosphate deficiency, nitrite starvation, or ambient temperature (16ºC) (Hsieh et al., 2009; Lee et al., 2010), suggesting that miR156 is an integrator of various nutritional signals. To execute the coordinative role, one mechanism is to assign various nutrition-responsive elements on a single *MIR156* promoter; an alternative approach would be to evolve paralogue genes that can respond to different nutritional signals independently. Either of these two approaches will result in a change in the mature

miR156 pool. It will be interesting to test which *MIR156* loci changes in responding to the phosphate and nitrite level.

4. A BAH DOMAIN CONTAINING PROTEIN PROMOTES MIRNA MEDIATED TRANSLATION REPRESSION

4.1. Abstract

Plant microRNAs (miRNAs) typically mediate RNA cleavage, but examples of miRNA-mediated translational repression have also been reported. The functional significance of this process for plant development is unclear. We identified *SUO* in a screen for *Arabidopsis* mutations that increase the expression of the miR156-regulated gene, *SPL3*. *suo* has a loss-of-function phenotype characteristic of plants with reduced AGO1 activity. An analysis of RNA and protein levels in *suo* mutants demonstrated that this phenotype is a consequence of a defect in miRNA-mediated translational repression; the effect of *suo* on vegetative phase change is specifically attributable to a reduction in miR156 activity. *SUO* encodes a large protein with N-terminal BAH and TSF2N domains and two C-terminal GW repeats. SUO is present in the nucleus, and co-localizes with the Processing-body (P-body) component DCP1 in the cytoplasm. Our results suggest that SUO is a functional homolog of the translational repressor GW182, and demonstrate that translational repression is important for the biological function of miRNAs in plants.

4.2. Background

miRNAs are important regulators of gene expression throughout eukaryotes. In animals, miRNAs repress gene expression by inhibiting translation and by promoting mRNA degradation (Eulalio et al., 2008; Fabian et al., 2010). In plants, miRNAs primarily mediate RNA cleavage (Llave et al., 2002; Kasschau et al., 2003; Palatnik et al., 2003). Some plant miRNAs also promote translational repression, but extent and functional significance of this process is still unknown.

Evidence that miRNAs repress translation in plants emerged soon after their discovery. One of the first miRNAs to be identified, miR172, targets the transcription factor AP2 (Park et al., 2002; Rhoades et al., 2002). Aukerman and Sakai (Aukerman and Sakai, 2003) found that over-expressing miR172 decreases the abundance of the AP2 protein without affecting the abundance of the AP2 mRNA, while Chen (Chen, 2004) reported that mutations that reduce miR172 levels increase the abundance of the AP2 protein without affecting AP2 mRNA. Subsequently, it was reported that over-expressing the miR156 target, SPL3, produced an increase in the SPL3 transcript without producing a corresponding increase in the SPL3 protein (Gandikota et al., 2007). Additional evidence for miRNA-mediated translational repression in plants comes from the discovery that mutations in the microtuble-severing protein, KATNIN (KTN), the cap-binding protein, VARICOSE (VCS), and the Argonaute protein, PNH/ZLL/AGO10, increase the proteins produced by some miRNA-regulated genes without causing a corresponding increase in the abundance of their mRNAs (Brodersen et al., 2008; Beauclair et al., 2010). The observation that AGO1-miRNPs are associated with

polysomes in Arabidopsis provide additional support for the conclusion that miRNAs repress translation in plants (Lanet et al., 2009).

Although there is no doubt that miRNA-mediated translational repression occurs in plants, its functional significance remains to be determined. The miRNAs that mediate translational repression also cause transcript cleavage (Llave et al., 2002; Kasschau et al., 2003; Sunkar et al., 2006; Wu and Poethig, 2006; Beauclair et al., 2010), making it difficult to distinguish the relative importance of these processes. Furthermore, there is still no evidence that the morphological and physiological phenotypes of *ktn*, *vcs* and *ago10* mutants can be attributed to a defect in miRNA-mediated translational repression. Mutations in *KTN* have effects on shoot and root morphology that have been attributed to defects in cytoskeletal organization and cell wall structure (Burk et al., 2001; Burk and Ye, 2002; Webb et al., 2002; Bouquin et al., 2003). *vcs* mutations have a seedling lethal phenotype that likely results from the widespread effect of this mutation on mRNA stability (Deyholos et al., 2003; Xu et al., 2006; Goeres et al., 2007). *pnh*/*zwl*/*ago10* mutations have a poorly penetrant phenotype. In a Landsberg *erecta* background, *pnh*/*zwl*/*ago10* mutations affect leaf polarity about 30% of the time (McConnell and Barton, 1995; Moussian et al., 1998; Lynn et al., 1999), but in a Columbia genetic background the vast majority of mutant plants (>99%) are morphologically normal (Mallory et al., 2009). Interestingly, the phenotype of *pnh*/*zwl*/*ago10* mutants has been attributed to an increase in the level of miR165/miR166, rather than to a decrease in miRNA activity (Liu et al., 2009).

Here, we describe the identification of *SUO*, a novel gene required for miRNA-mediated translational repression in Arabidopsis. Loss-of-function mutations in *SUO* were identified in a screen for mutations that affect vegetative phase change. In addition to accelerating the expression of adult traits, *suo* mutants have a variety of defects characteristic of genes required for miRNA biogenesis or function. We show that the phase change phenotype of *suo* can be attributed to a defect in the function of miR156. A comparison of the effect of *suo* on the protein and mRNA products of miRNA-regulated genes demonstrates that *suo* interferes with the translational repression function of miRNAs, but not their cleavage activity. Furthermore, the observation that SUO localizes in P-bodies suggests that—as in animals—these cytoplasmic structures are the primary location for miRNA-mediated translational repression. Our results reveal a new component of the translational repression machinery in plants and demonstrate that this process plays an important role in miRNA-mediated regulation of plant development.

4.3.Results

4.3.1. Identification of suo

miR156 is highly expressed early in shoot development, and promote the juvenile traits by repressing the expression of 10 members of the SPL transcription factor family in *Arabidopsis* (Schwab et al., 2005; Wu and Poethig, 2006; Gandikota et al., 2007). Mutations that interfere with the biogenesis of miR156 or reduce the activity of AGO1 cause an increase of SPL transcripts (Vaucheret et al., 2004; Vazquez et al., 2004; Park et al., 2005; Ronemus et al., 2006; Smith et al., 2009) and accelerate the expression of adult vegetative traits (Telfer et al., 1997; Berardini et al., 2001; Wu et al., 2009). Mutations that render SPL genes insensitive to miR156 have a precocious phenotype similar to that produced by loss of miR156 activity (Wu and Poethig, 2006; Shikata et al., 2009; Usami et al., 2009; Wu et al., 2009).

To identify genes required for the expression and/or action of miR156, we screened for mutations that enhance the expression of the miR156-regulated reporter pSPL3::eGFP-SPL3. This construct consists of a 4 kb genomic fragment that includes *SPL3* and its 5' and 3' flanking regions, with eGFP inserted upstream of the start codon of *SPL3* (Figure 4.1 A). Plants transformed with pSPL3::eGFP-SPL3 had no detectable GFP in leaves 1 and 2, but expressed GFP increasingly brightly in subsequent leaf primordia and fully-expanded leaves (Figure 4.1 B, C). GFP expression was localized to the nucleus, as expected from the evidence that SPL3 is a transcription factor (Figure 4.1

B, C) (Cardon et al., 1997). pSPL3::eGFP-SPL3 seeds were mutagenized with EMS and the M2 progeny of

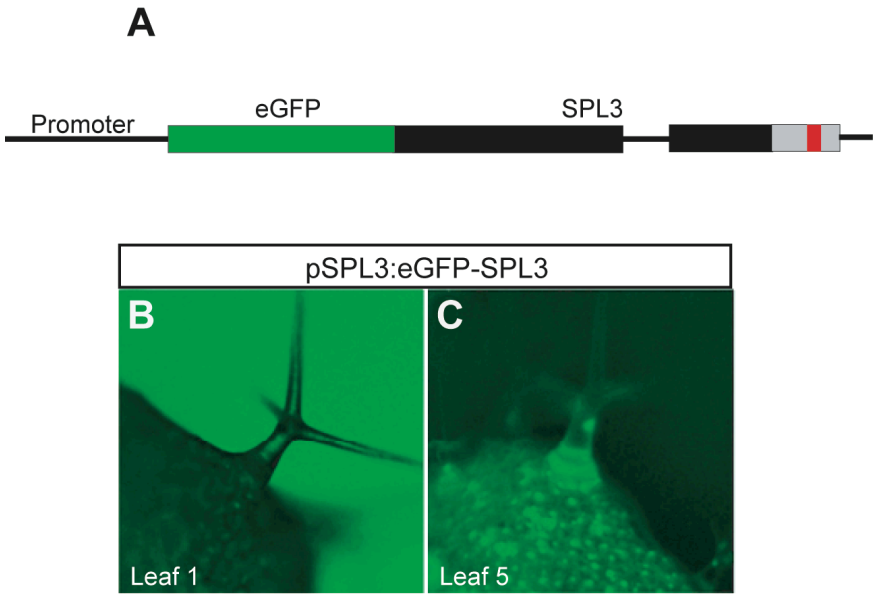


Figure 4.1: A translational reporter for SPL3

A) Structure of the SPL3 reporter. A eGFP coding region is inserted upstream of SPL3 start codon. B) and C) Temporal expression of eGFP-SPL3. eGFP is not expressed in leaf 1 (B), and highly expressed in leaf 5 (C).

these plants were screened under a stereomicroscope for seedlings with elevated GFP expression. miR156 and miR159 level was examined in each *see* mutants (*see*: SPL3 eGFP enhancer), and mutants were classified into four categories based on the expression of miR156 and miR159 (see Appendix Figure 8.1). The class I mutants are with reduced miR156 and normal miR159 (e.g. *see25*), indicating defects in miR156 expression; the class II mutants are with reduced miR156 and miR159 (e.g. *see38*), indicating defects in microRNA biogenesis; The class III mutants have elevated level of miR156 and miR159 (e.g. *see243*), indicating defects in microRNA action. In class IV, mutants do not change the abundance of miR156 or miR159 (e.g. *see219*). A summary of *see* mutants is in Table 8.1.

One mutant identified in the Class III had elevated GFP expression in leaf 5, and also had more serrated leaves (Figure 4.2 A, B). We named this mutant *suo-1* (meaning "shuttle" in Chinese) to reflect its precocious phenotype. Several additional alleles of *SUO* were subsequently identified in our laboratory and in the SALK T-DNA insertion collection (see below). These alleles are morphologically indistinguishable from *suo-1*.

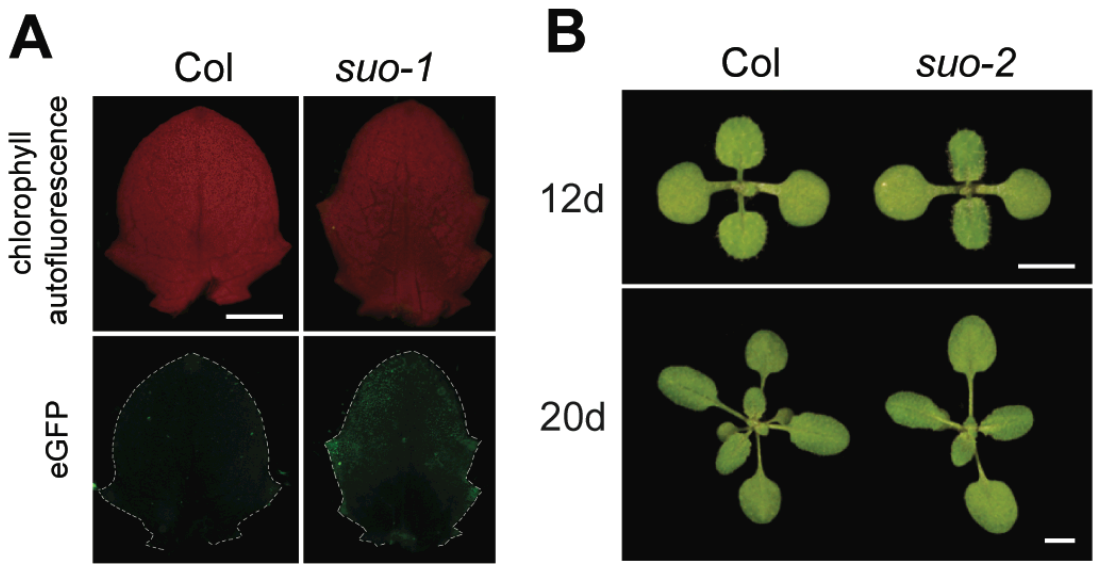


Figure 4.2 Identification of *suo*

A) The fifth leaf of pSPL3::eGFP-SPL3 and *suo-1* pSPL3::eGFP-SPL3 plants. *suo-1* increases the fluorescence of pSPL3::eGFP-SPL3 and has serrated leaf primordia. Scale bar = 1 mm. B) 12- and 20-day-old wild-type Columbia (Col) and *suo* mutants. *suo* mutants have a reduced rate of leaf initiation, and produce elongated leaves starting with leaf 3. Scale bar = 5 mm.

To determine if *SUO* is specifically required for vegetative phase change, we conducted a careful analysis of its mutant phenotype. *suo* mutants are most readily identifiable early in development by their slow rate of rosette development and their slightly enlarged first two leaves (Figure 4.3 A). Under long day (LD) conditions, *suo-2* produced significantly fewer juvenile leaves than wild-type plants: mutant plants produced 3-4 leaves lacking abaxial trichomes (3.2 ± 0.6, n=24), whereas wild-type plants produced 4 or 5 (4.3 ± 0.4, n=24) (Figure 4.3 A). Consistent with this effect on abaxial trichome production, the fully expanded rosette leaves of mutant plants were more serrated than normal and displayed the elongated shape and short, thick petiole typical of adult leaves (Figure 4.3 A). The siliques of mutant plants were sometimes produced in an irregular spacing pattern (Figure 4.3 B, C), or fused at the base (Figure 4.3 D). In addition to these developmental phenotypes, *suo* was more drought-resistant than wild-type plants: 96% of wild-type plants withered (n=25) after being exposed in drought stress for two weeks, compared to only 12% of *suo-2* plants (n=20) (Figure 4.3 E). Because drought resistance is often associated with a change in ABA sensitivity, we tested the effect of ABA on seed germination in mutant and wild-type plants. Consistent with their drought-resistant phenotype, *suo* mutants were hypersensitive to ABA. 80% of wild-type seeds germinated in the presence of 1 uM ABA, compared to only 40% of *suo-2* and 0% of *suo-3* seeds (n=120 for each genotype) (Figure 4.3 F). *suo* mutants also produced significantly fewer rosette leaves (7.4 ± 0.7, n=24) than wild-type (10.1 ± 0.6, n=24), both because of their reduced rate of leaf initiation, and because they stopped producing leaves earlier than normal (Figure 4.3 G). Thus, *SUO* is required for a wide range of biological processes. Furthermore, all of alleles we tested had a semi-dominant

effect on abaxial trichome production and the rate of leaf initiation (Figure 4.4 A, B), suggesting that *SUO* is haplo-insufficient.

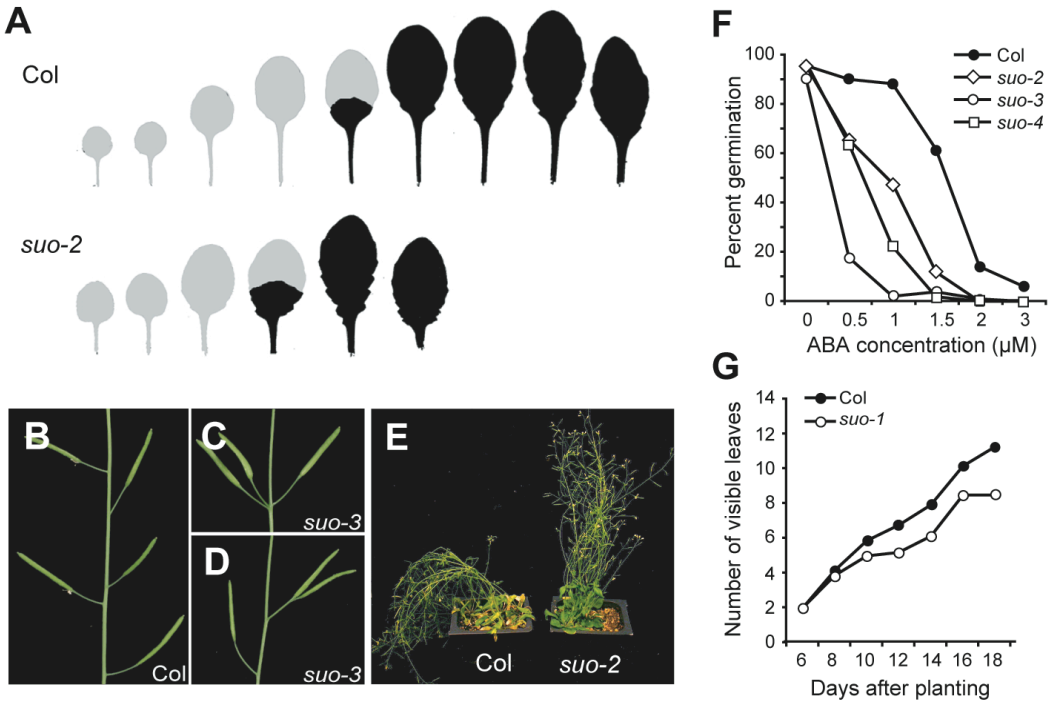


Figure 4.3: ***suo*** **has a pleiotropic phenotype**

A) Fully expanded rosette leaves of Col and *suo-2*. Numbers indicate the leaf position on the shoot. Gray indicates leaves lacking abaxial trichomes, and black indicates leaves with abaxial trichomes. B-C) suo exhibits disrupted silique patterning. In Col (B), the siliques are organized in regular intervals. In different *suo* alleles we observed multiple siliques clustered at one node (C) and fused siliques (D). E) *suo* mutant is resistant to drought. Most *suo* mutants survived two weeks without water. F) *suo* is hypersensitive to ABA. *suo* mutants have a lower germination rate on ABA-containing plates than wild type. G) The rate of leaf initiation is reduced in *suo*.

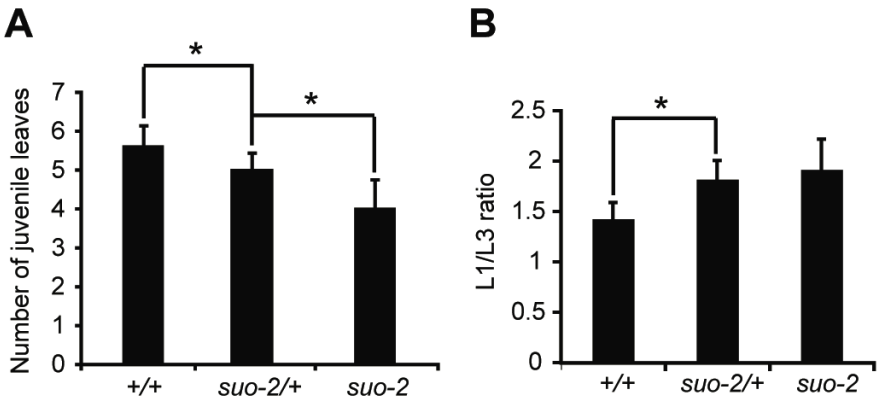


Figure 4.4: *suo* is haplo-insufficient

A) The number of leaves without abaxial trichomes in *suo-2*/+ plants is intermediate between +/+ and *suo-2*/*suo-2* plants. Asterix = significantly different ($p < 0.01$, $n = 24$) B) The ratio of the length of leaf 1 to leaf 3 in 16 day-old plants is greater in *suo-2* than in +/+ because of the delay in leaf initiation in *suo-2*. This ratio is significantly greater in *suo-2*/+ plants than in +/+ plants ($p < 0.01$, $n = 10$).

4.3.2. SUO encodes a novel GW-containing protein

Map-based cloning revealed that *suo-1* is a point mutation in At3g48050 that converts a glycine to an arginine at position 324 (Figure 4.5 A). *suo-2* is a 14 bp deletion in At3g48050 (nucleotides 2041-2054 of the coding sequence); the resulting change in reading frame introduces a stop codon immediately downstream of the deletion (Figure 2A). Four additional T-DNA insertion alleles (*suo-3* to *suo-6*) were obtained from the ABRC (Figure 4.5 A), and were found to have a phenotype identical to *suo-1* and *suo-2*. Quantitative RT-PCR (qRT-PCR) revealed that *suo-2* and *suo-3* significantly reduce the abundance of the SUO transcript (Figure 4.5 C). This result, and the observation that all of these alleles have the same morphological phenotype, suggests that this phenotype represents the null, or nearly null, phenotype of At3g48050. Constitutive expression of SUO under 35S promoter rescued the mutant phenotype of *suo-2*, confirming that this gene corresponds to defects in *suo* (Figure 4.5 D).

The primary transcript of At3g48050 contains 4 exons and encodes a 1,613 amino acid protein that is conserved throughout plants—including the moss *Physcomitrella patens*—but has no close relative in animals (Figure 4.5 B). The most highly conserved part of the protein is its N-terminal end, which possesses a Bromo-adajacent homology (BAH) domain and a region with similarity to the transcription elongation factor S-II (TFS2N). The central part of the protein has no recognizable domains and is poorly conserved between species. The C-terminal part of the protein contains two highly-conserved GW-containing sequences (Figure 4.5 B). GW repeats are

found in proteins involved in siRNA- and miRNA-mediated processes and are required for the interaction of these proteins with Argonaute proteins. GW-repeat proteins include the mammalian protein, GW182 (Eulalio et al., 2009a), the *C. elegans* proteins, AIN1 and AIN2 (Ding et al., 2005; Ding and Grosshans, 2009), the plant proteins, NRPE1 (El-Shami et al., 2007), KTF1/RDM3/SPT5-like (Bies-Etheve et al., 2009; He et al., 2009), and WGRP1 (Karlowski et al., 2010), and the plant viral proteins, P1 and P38 (Azevedo et al., 2010; Giner et al., 2010). In addition to these domains, SUO contains 5 regions that share the amino acid sequence L/FDLN and are rich in the negatively charged amino acids aspartic acid (D) and glutamic acid (E). This sequence closely resembles the EAR/DLN transcriptional repressor motif L/FDLNL/F(x)P (Ohta et al., 2001).

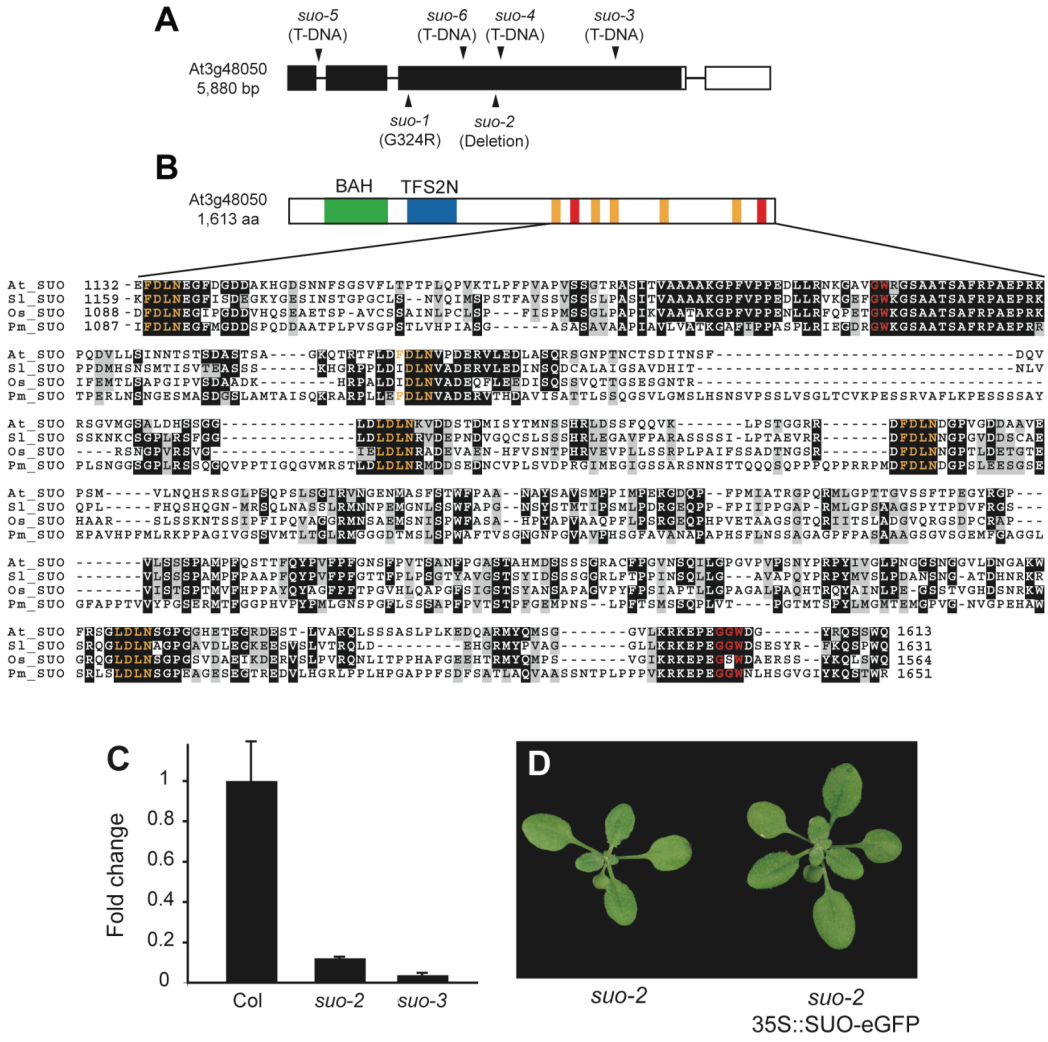


Figure 4.5: *SUO* Encodes a Novel GW-containing Protein

A) Genomic structure of At3g48050, and the location and nature of *suo* alleles. Black box = exon; line=intron; open box =UTR. B) The domain organization of the At3g48050 protein (At, Genbank NP_850669), based on an alignment with similar predicted proteins from tomato (*Solanum lycopersicum*, Sl; Genbank AAX95757), rice (*Oryza sativa*, Os; Genbank EEE57790), and moss (*Physcomitrella patens*, Ps; EDQ76943). In addition to a conserved N-terminal bromo-adjacent homology domain (BAH) and a TFS2N domain, the C-terminal end of the protein contains two highly conserved GW-containing sequences (red), and 5 repeats of the sequence L/FDLN (orange). C) The mRNA level of AT3G48050 is reduced in *suo* alleles. *ACTIN* is an endogenous control. D) 35S:SUO-eGFP rescues *suo-2* phenotype. The slow leaf initiation and elongated leaf phenotype are rescued in transgenic plants. (Scale bar=5mm)

SUO has a closely-related paralogue (95% amino acid identity), At3g48060, located 3.6 kb from its 5' end (Figure 4.6 A). Although qRT-PCR revealed that this gene is expressed at nearly the same level as *SUO*, an RNA-null mutation of At3g48060 (SALK_086029) had no obvious morphological defects (Figure 4.6 B, C).

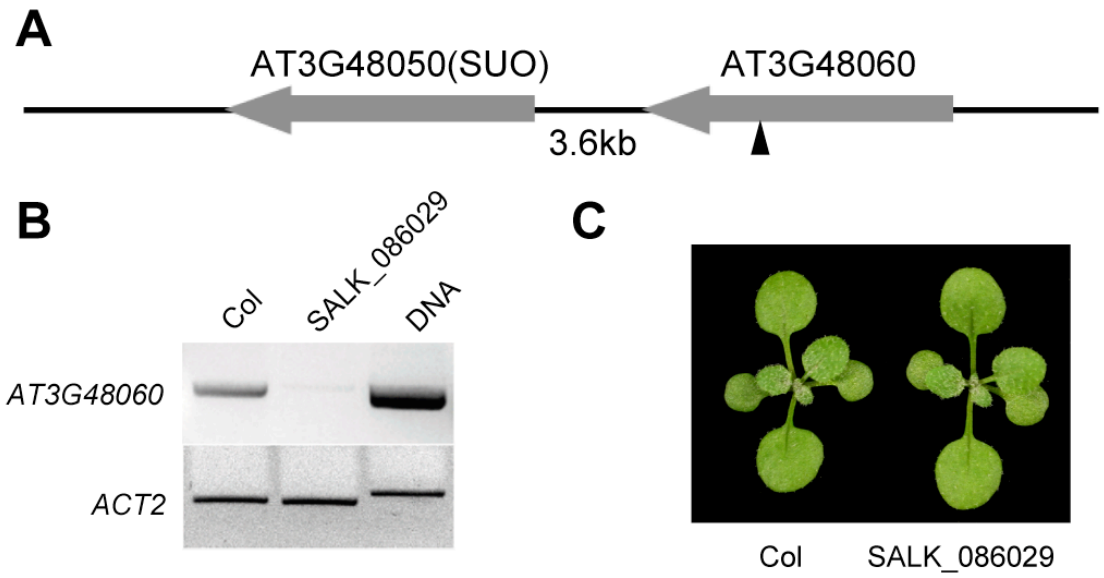


Figure 4.6: At3g48060 has no obvious mutant phenotype

A) The genomic organization of of SUO (At3g48050) and the closely related gene At3g48060. B) RT-PCR analysis of 14-day-old seedlings demonstrates that the SALK_086029 insertion eliminates the At3g48060 mRNA. C) Plants homozygous for SALK_086029 have no obvious morphological phenotype. Arrowhead in (A) indicates the position of SALK_086029.

4.3.3. The vegetative phenotype of suo is attributable to a defect in miR156 function

The constellation of defects displayed by *suo* is characteristic of mutants that disrupt miRNA biogenesis or function. To explore the possibility that *SUO* is involved in these processes, we generated double mutants between *suo* and mutations that affect miRNA biogenesis (*abh1, se*), miRNA export/stability (*hst*), and miRNA activity (*sqn*, *ago1*) (Voinnet, 2009). *suo* interacted with all of these mutants, but to different extents (Figure 4.7). *suo-2* interacted relatively weakly with *ago1-45* and *sqn-1,* both of which reduce AGO1 activity (Smith et al., 2009). Under short day (SD) conditions, *suo-2 sqn-1* and *suo-2 ago1-45* double mutants produced one less leaf lacking abaxial trichomes and had a slightly stronger leaf shape phenotype than either single mutant (Figure 4.7 A-F). *suo-2* interacted more strongly with *hst*, *abh1* and *se*. Although double mutants had only a modestly more severe abaxial trichome phenotype, they were significantly smaller than the single mutants, and displayed the up-curled leaf phenotype typical of mutants with severe defects in miRNA activity (Figure 4.7 G-L). These results suggest that SUO acts in association with SQN and/or AGO1, and independently of ABH, SE or HST.

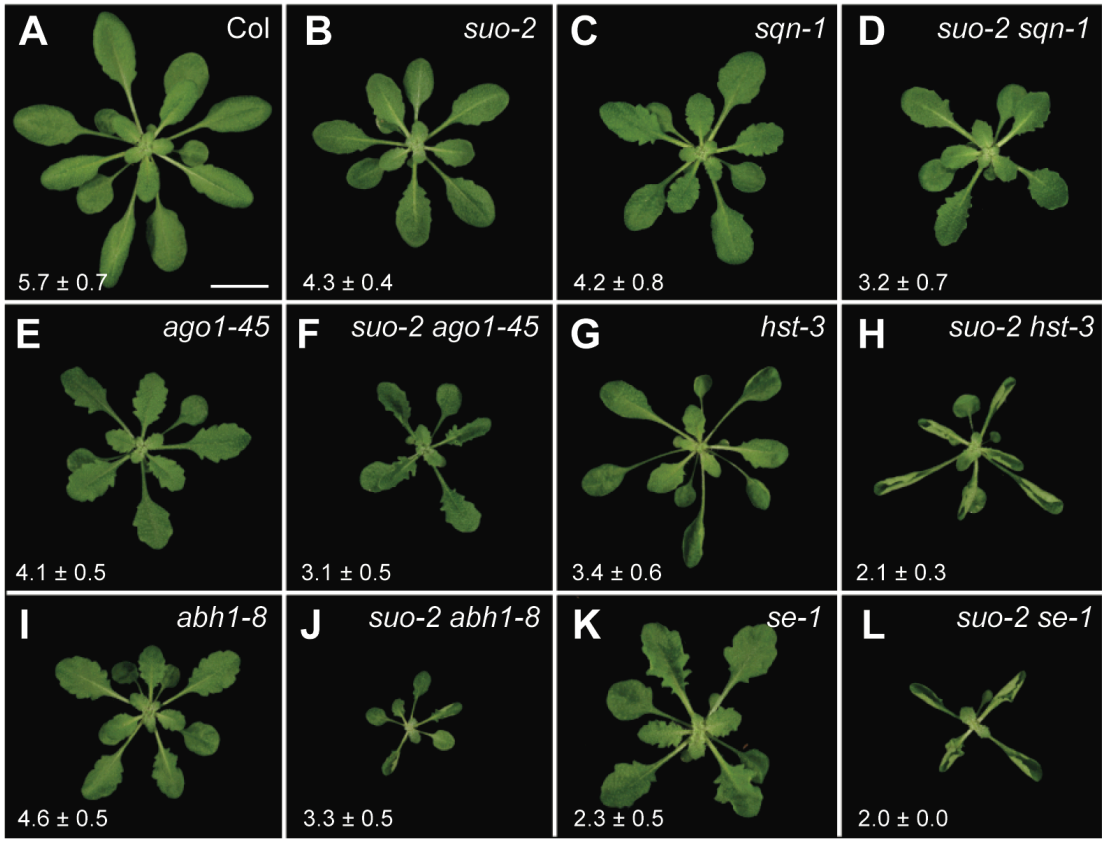


Figure 4.7: *suo* interacts genetically with mutants in the miRNA pathway

The morphology of the rosettes of one-month-old Col (A) and mutant (B-L) plants grown in SD. *suo-2* interacts more strongly with *hst-3* (G, H), *abh1-8* (I, J), and *se-1* (K, L) than with *sqn-1* (C, D) and *ago1-45* (E, F). The numbers represent the number of juvenile leaves. Scale bar = 1 cm.

Constitutive over-expression of miR156 under the regulation of the 35S promoter delays vegetative phase change and flowering and accelerates leaf initiation (Schwab et al., 2005; Wu and Poethig, 2006; Wang et al., 2008; Wang et al., 2009a) (Figure 4.8 A), which is the exact opposite of the *suo* phenotype. To test the hypothesis that *suo* reduces the activity of miR156, we examined the interaction between *suo* and 35S::MIR156A. 35S::MIR156A *suo-2* plants had 6 fewer juvenile leaves (Figure 4.8 A) and a slower rate of leaf initiation (Figure 4.8 B) than 35S::MIR156A transgenic plants, supporting this hypothesis. As an additional test of this hypothesis, we generated a *suo-2 spl9-4 spl15-1* triple mutant. *SPL9* and *SPL15* are direct targets of miR156 and produce a phenotype similar to that of *suo* when over-expressed (Usami et al., 2009; Wu et al., 2009); loss-of-function mutations of these genes have prolonged juvenile phase (Schwarz et al., 2008). If the phenotype of *suo* is attributable to an increase in the expression of these genes, *spl9* and *spl15* loss-of-function mutations should suppress the phenotype of *suo*. *suo-2 spl9-4 spl15-1* triple mutants had a phenotype intermediate between that of *suo-2* and *spl9-4 spl15-1* (Figure 4.8 C). Triple mutants produced abaxial trichomes 2 leaves earlier than *spl9-4 spl15-1*, but 4 leaves later than *suo-2*, and their leaf morphology was intermediate as well. In addition, *suo-2 spl9-4 spl15-1* plants had an intermediate rate of leaf initiation, which was indistinguishable from that of wild-type plants (Figure 4.8 D). Thus, loss of SPL9 and SPL15 partially corrects some aspects of the *suo* phenotype. These results are consistent with the elevated expression of eGFP-SPL3 in *suo* mutants (Figure 4.1 A), and suggest that the phase change defects of *suo* are attributable to an increase in the expression of SPL genes.

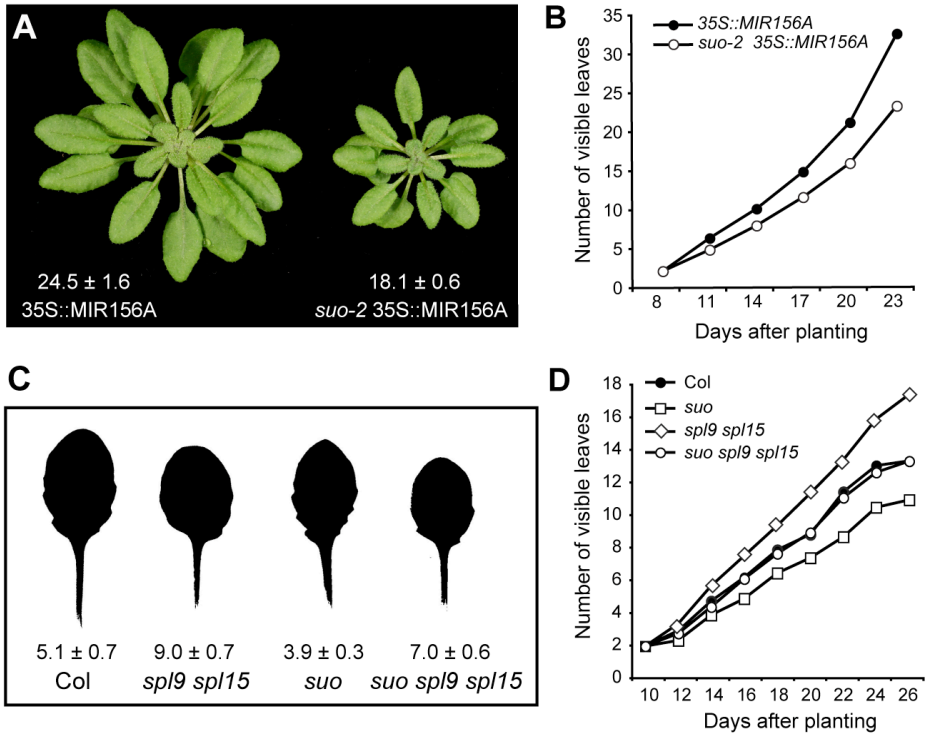


Figure 4.8: *suo* interferes with the function of *miR156.*

A) The morphology of 35S::MIR156A and *suo-2* 35S::MIR156A plants grown in long days. The number of leaves lacking abaxial trichomes is indicated (± s. d.), and demonstrates that *suo-2* partially suppresses the effect of 35S::MIR156A. B) The rate of leaf initiation of 35S::MIR156A and *suo-2* 35S::MIR156A plants grown in long days. *suo-2* suppresses the increase in the rate of leaf initiation produced by 35S::MIR156A. C) The morphology of 5th leaf, and the number of leaves lacking abaxial trichomes (± s.d.) in Col and *spl9-4 spl15-1*, *suo-2*, and *suo-2 spl9-4 spl15-1* plants. The phenotype of the triple mutant is intermediate, but more closely resembles *spl9 spl15* than *suo-2*. D) The rate of leaf initiation in Col and *spl9-4 spl15-1*, *suo-2*, and *suo-2 spl9-4 spl15-1* plants grown in LD The rate of leaf initiation in the triple mutant is intermediate between the parental genotypes.

4.3.4. SUO is not required for miRNA biogenesis or miRNA-mediated transcript cleavage

The genetic data indicate that SUO interacts with miRNA pathway. To define the molecular function of SUO, we examined the effect of *suo* mutations on the abundance of mature miRNAs and their precursor transcripts. RNA blots of 14-day-old seedlings revealed that mature miRNAs were largely unaffected (miR156, miR161, miR164, miR398) or elevated (miR159, miR165/miR166, miR167, miR169, miR171, miR172) in *suo* mutants (Figure 4.9 A). *suo-3* and *suo-4* had a slightly stronger effect than *suo-2*. qRT-PCR revealed that the abundance of miRNA precursors was correlated with the abundance of the mature miRNA, suggesting that the increase in mature miRNA levels is a consequence of increased transcription of their precursors (Figure 4.9 B). We examined the effect of *suo* on miRNA activity by measuring the abundance of transcripts directly regulated by various miRNAs. qRT-PCR demonstrated that, with the exception of *CUC2*, there was no significant difference in the abundance of these transcripts in mutants and wild-type plants (Figure 4.9 C). To determine if SUO is required for miRNA-directed RNA cleavage, we used RNA ligase mediated rapid amplification of 5' cDNA ends (RLM-RACE) (Liu and Gorovsky, 1993) to test for the presence of the expected cleavage fragments in *suo-2* and *suo-3*. Consistent with the observation that these mutations have no effect on overall abundance of miRNA-regulated transcripts, the abundance of these cleavage fragments was approximately the same in mutant and wild type plants (Figure 4.9 D). We conclude that SUO is not required for miRNA biogenesis or stability, and is also dispensable for miRNA-mediated transcript cleavage.

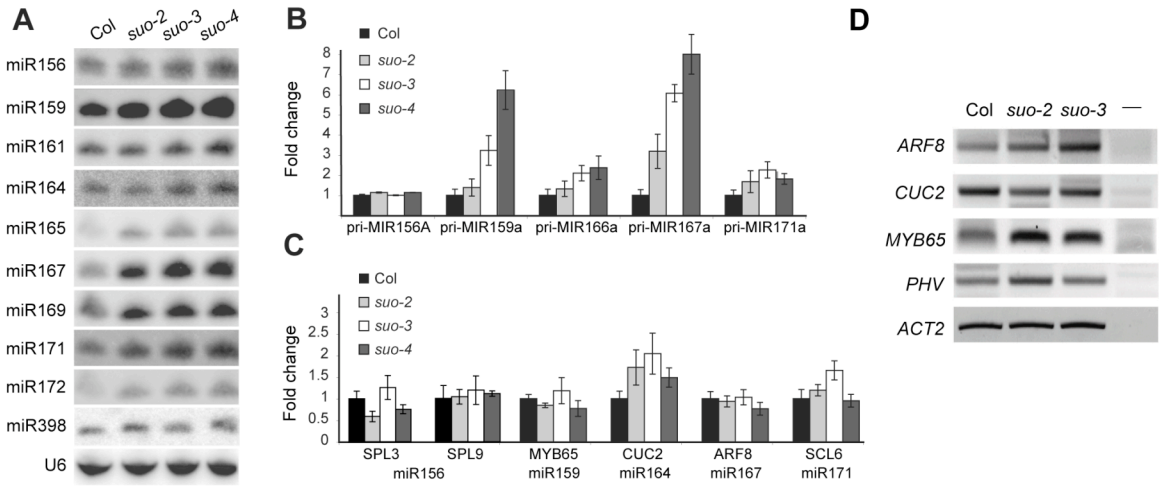


Figure 4.9: Accumulation of miRNAs and their targets in *suo*

A) RNA blot of small RNA from 14-day seedlings of Col and *suo* mutants, sequentially hybridized with probes for various miRNAs. The level of mature miRNAs is either unchanged or slightly elevated in *suo*. B) qRT-CR analysis of the abundance of the primary transcripts of miRNAs in 14-day seedlings of Col and *suo* mutants (Bars = ± s.d. of 3 technical replicates) C) qRT-CR analysis of miRNA-regulated transcripts in 14-day seedlings of Col and *suo* mutants. The miRNA targeting each transcript is indicated. D) RLM-5'RACE of the transcripts of miRNA-targetted genes reveals fragments of the expected size for each gene.

4.3.5. SUO promotes miRNA-mediated translational repression

suo-1 was originally identified because it increases the fluorescence of the eGFP-SPL3 protein (Figure 4.2 A). qRT-PCR revealed no significant difference in the abundance of the eGFP-SPL3 transcript in *suo-1* and wild -type plants (Figure 4.10 A), but western blots probed with an antibody to GFP confirmed that the eGFP-SPL3 protein is elevated in the third and fourth leaf of *suo-1* (Figure 4.10 B). This result suggests that SUO is required for the translational repression of *SPL3*. To determine if SUO is required for the translational repression of other miRNA-regulated genes, we examined the effect of *suo* on the products of *CSD1* and *CSD2*—genes that are translationally repressed by miR398 under low copper conditions (Brodersen et al., 2008). *suo* mutants had normal levels miR398 (Figure 4.9 A) and the *CSD1* and *CSD2* mRNAs (Figure 4.10 C), but had increased levels of CSD1 and CSD2 proteins (Figure 4.10 D). We conclude that SUO promotes the miRNA-mediated translational repression of *SPL3*, *CSD1* and *CSD2*.

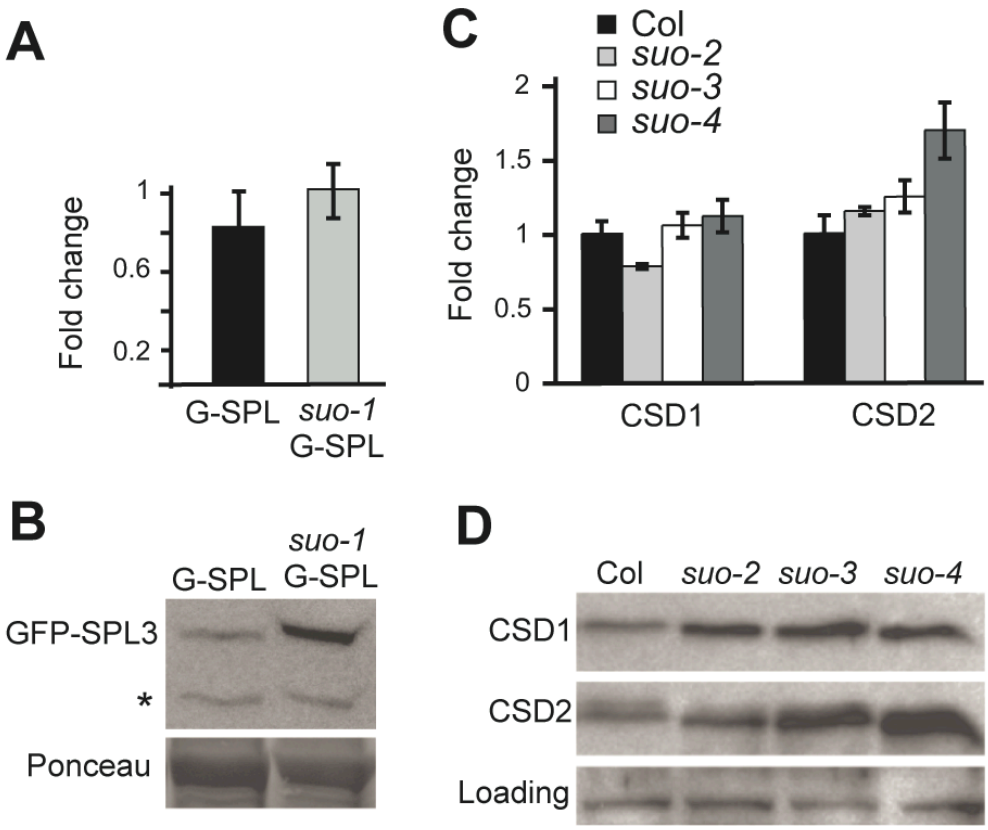


Figure 4.10: SUO is required for miRNA-mediated translational repression

A) qRT-PCR analysis of the eGFP-SPL3 mRNA in leaf 3 and 4 (approximately 0.5 cm long) of pSPL3::eGFP-SPL3 and *suo-1* pSPL3::eGFP-SPL3 plants (± s. d.). *suo-1* has no significant effect on the level of the eGFP-SPL3 transcript. B) Western blot of pSPL3::eGFP-SPL3 and *suo-1* pSPL3::eGFP-SPL3 leaf 3 and 4 probed with an antibody to GFP. The eGFP-SPL3 protein is elevated in *suo-1*. The asterix indicates a non-specific band that serves as a loading control. Ponceau staining confirms that the lanes were loaded evenly. C) qRT-PCR analysis of the CSD1 and CSD2 mRNAs in *suo-2*, *suo-3* and *suo-4* plants. The level of these transcripts is not significantly different in mutant and Col plants. D) Western blot of Col and mutant plants probed with an antibody to CSD1 and CSD2. Both of these proteins are elevated in *suo* mutants.

Processing bodies (P-bodies) are cytoplasmic structures that have been implicated in miRNA-mediated translational repression and mRNA degradation (Xu and Chua; Franks and Lykke-Andersen, 2008; Fabian et al., 2010). To determine the sub-cellular location of SUO, we produced transgenic *Arabidopsis* plants expressing 35S::SUO-eGFP; this transgene rescued the mutant phenotype of *suo-2* (Figure 4.4 B), demonstrating that the SUO-eGFP protein is functional. SUO-eGFP was strongly expressed in the nucleus in Arabidopsis root cells (Figure 4.11 A) and in *Nicotiana benthamiana* epidermal cells (Figure 4.11 B), and was also present in cytoplasmic foci that resembled P-bodies in size and number. We also co-infiltrated the 35S::SUO-eGFP construct into *Nicotiana benthamiana* leaves along with pDCP1::DCP1-CFP; DCP1 promotes the activity of the decapping enzyme DCP2, and is found exclusively in P-bodies (Xu et al., 2006). SUO-eGFP expression over-lapped with DCP1-CFP expression in cytoplasmic foci in *N. benthamiana* cells (Figure 4.11 C-E), indicating that they are indeed P-bodies. To determine if SUO is required for P-body assembly, we transformed wild-type and *suo-2* plants with pDCP1::DCP1-CFP. There was no obvious difference in the size, structure, or number of CFP-expressing bodies in these genotypes (Figure 4.11 F-H), indicating that SUO is not essential for P-body formation.

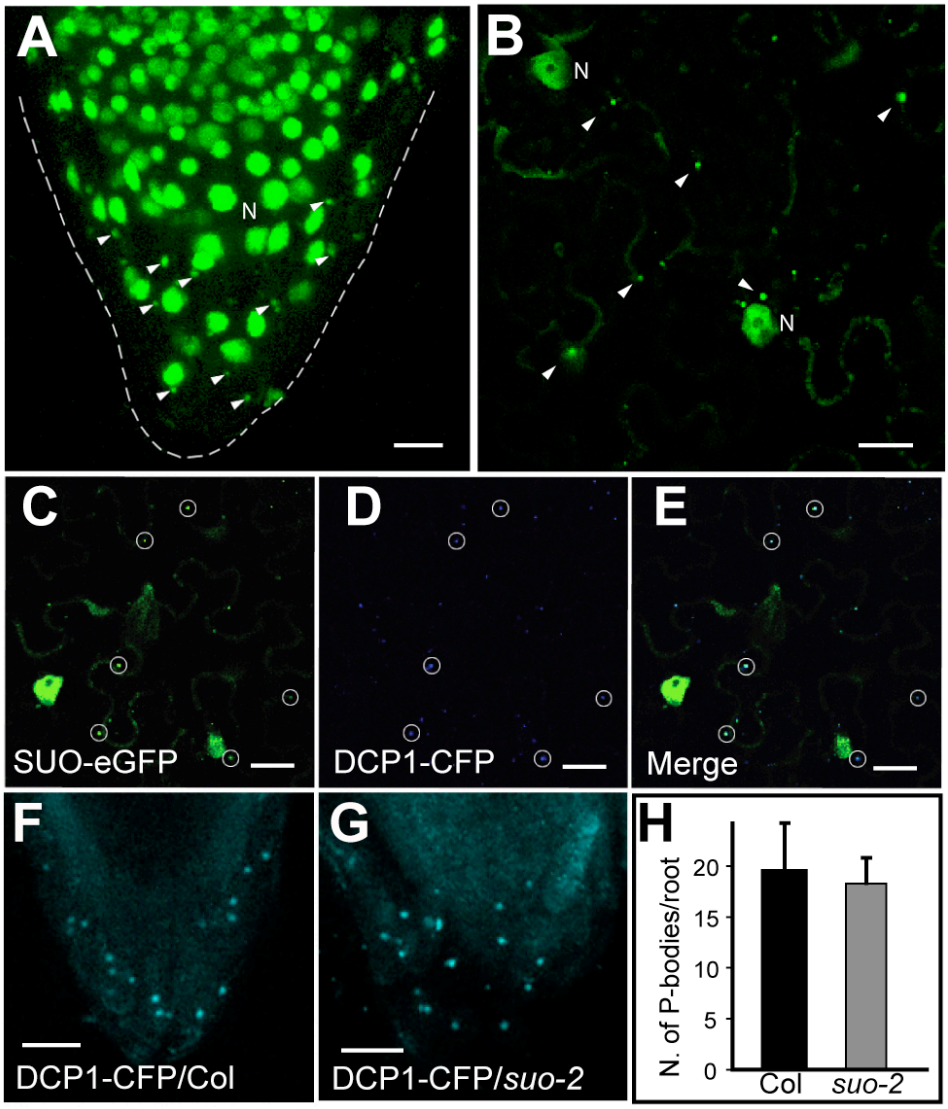


Figure 4.11: SUO is present in the nucleus and in P-bodies.

A) Confocal images of eGFP fluorescence in the root apex of a 3-day-old *Arabidopsis* seedling and B) an epidermal cell of *a N. benthamiana* leaf transformed with 35S::SUO-eGFP. Fluorescence is evident in nuclei (N) and in cytoplasmic foci (arrowheads). C-E) Subcellular localization of SUO and DCP1 in tobacco epidermal cells co-transformed with 35S::SUO-eGFP and pDCP1::DCP1-CFP. CFP fluorescence is pseudo-colored in blue and eGFP in green. Note that only SUO-eGFP is present in the nucleus. Some co-localized signals are highlighted by circles. F-G) The location of DCP1-CFP in root apical cells of wild-type and *suo-2* plants transformed with pDCP1::DCP1-CFP. H) The number of CFP-expressing bodies in the root apex of wild-type and *suo-2* plants transformed with pDCP1::DCP1-CFP. Scale bars = 10 μm in A, F and G, 20 μm in B, C, D and E.

4.4.Discussion

The results presented here indicate that SUO is a new component of the translation repression machinery in *Arabidopsis*, and suggest that it is specifically required for miRNA-mediated translational repression. Evidence that SUO promotes translational repression is provided by the observation that *suo* mutations increase the SPL3, CSD1 and CSD2 proteins without affecting the abundance of the mRNAs for these proteins, and by the presence of SUO in P-bodies—structures known to be involved in this process. We believe that SUO is likely to be specifically required for miRNA-mediated translational repression because of the phenotypic similarity between *suo* mutants and mutations in microRNA biogenesis and function, especially weak *ago1* alleles and *sqn*. It is also significant that *suo* interacts more strongly with mutations in miRNA biogenesis than with either *ago1* or *sqn* mutations. The simplest interpretation of this genetic result is that SUO operates independently of genes involved in miRNA biogenesis, and in association with AGO1.

The presence of two conserved GW domains in SUO provides strong support for this conclusion. GW/WG motifs are commonly found in proteins that interact with Argonaute (El-Shami et al., 2007; Eulalio et al., 2009b; Karlowski et al., 2010). In Arabidopsis, GW/WG-containing regions of the largest subunit of PolV, NRPE1 (El-Shami et al., 2007) and the transcription factor, KTF1/RDM3/SPT5-like (Bies-Etheve et al., 2009; He et al., 2009), mediate the interaction of these proteins with AGO4. Similarly, the plant viral proteins, P1 and P38, contain two GW domains that mediate their interaction with AGO1 (Azevedo et al., 2010; Giner et al., 2010). In mammals,

miRNA-directed translational repression and transcript degradation requires GW182, a protein that interacts with Ago2 via an N-terminal GW/WG domain (Eulalio et al., 2009b). In *C. elegans*, the function of GW182 is provided by the GW proteins, AIN1 and AIN2, which also promote miRNA-mediated translational repression and mRNA degradation via an interaction with Argonaute proteins (Ding et al., 2005; Ding and Grosshans, 2009). SUO is structurally different from these proteins, but is similar to GW182/AIN1/AIN2 proteins in that it promotes translational repression by miRNAs and is located in P-bodes. These similarities suggest that SUO is a functional analogue of GW182.

In addition to GW repeats, SUO possesses a BAH and a TFS2N domain, as well as several copies of the sequence L/FDLN. The BAH domain is commonly found in proteins that promote heterochromatin formation and gene silencing through either DNA methylation or histone modification (Callebaut et al., 1999). The TFS2N domain is found in the N-terminal end of transcription elongation factor S-II, a protein that increases the transcription rate of RNA polymerase II. L/FDLN is the core sequence of the DLN/EAR domain, a potent transcriptional repressor (Ohta et al., 2001). The predicted functions of these three domains suggest that SUO may repress transcription in addition to repressing translation. In this respect, it is interesting that although *suo* had no apparent effect on the transcription of miRNA-regulated genes, the primary transcripts of several miRNAs (pri-miRNAs), as well as the corresponding mature miRNAs, were elevated in *suo* mutants. This result raises the possibility that SUO directly or indirectly represses the transcription of at least some miRNA genes. If SUO specifically regulates the transcription of these genes, the question of how SUO is directed to miRNA genes

will need to be answered. One possibility is that it is directed to miRNA genes by components of the miRNA machinery. If so, this would reveal an unexpected involvement of these miRNA-related proteins in transcriptional regulation.

How important is translational repression for miRNA activity in plants? Null alleles of DCL1—the dicer that produces miRNAs in Arabidopsis—are lethal very early in embryogenesis (Schauer et al., 2002). By contrast, putative null alleles of *suo* have a relatively weak phenotype. We have no evidence that miRNA-mediated translational repression is completely absent in *suo* mutants, and this seems unlikely given the existence of the closely related *SUO* paralog At3g48060. On the other hand, the observation that *SUO* is haplo-insufficient whereas a null allele of At3g48060 has no obvious homozygous phenotype indicates that SUO is either much more important for this process than At3g48060, or that these genes are functional distinct. It may be that the phenotype of *suo* is in fact an accurate reflection of the contribution of translation repression to miRNA activity in plants. If so, the phenotype of *suo* suggests that this process is much less important for miRNA activity in plants than their role in transcript cleavage. The work described here demonstrates that translational repression is important for the biological function of at least one miRNA--miR156--and provides a foundation for future studies of the mechanism of this process.

5. CONCLUSION AND FUTURE DIRECTION

5.1. Conclusions

The results presented in this thesis are summarized in Figure 5.1. (Figure 5.1).

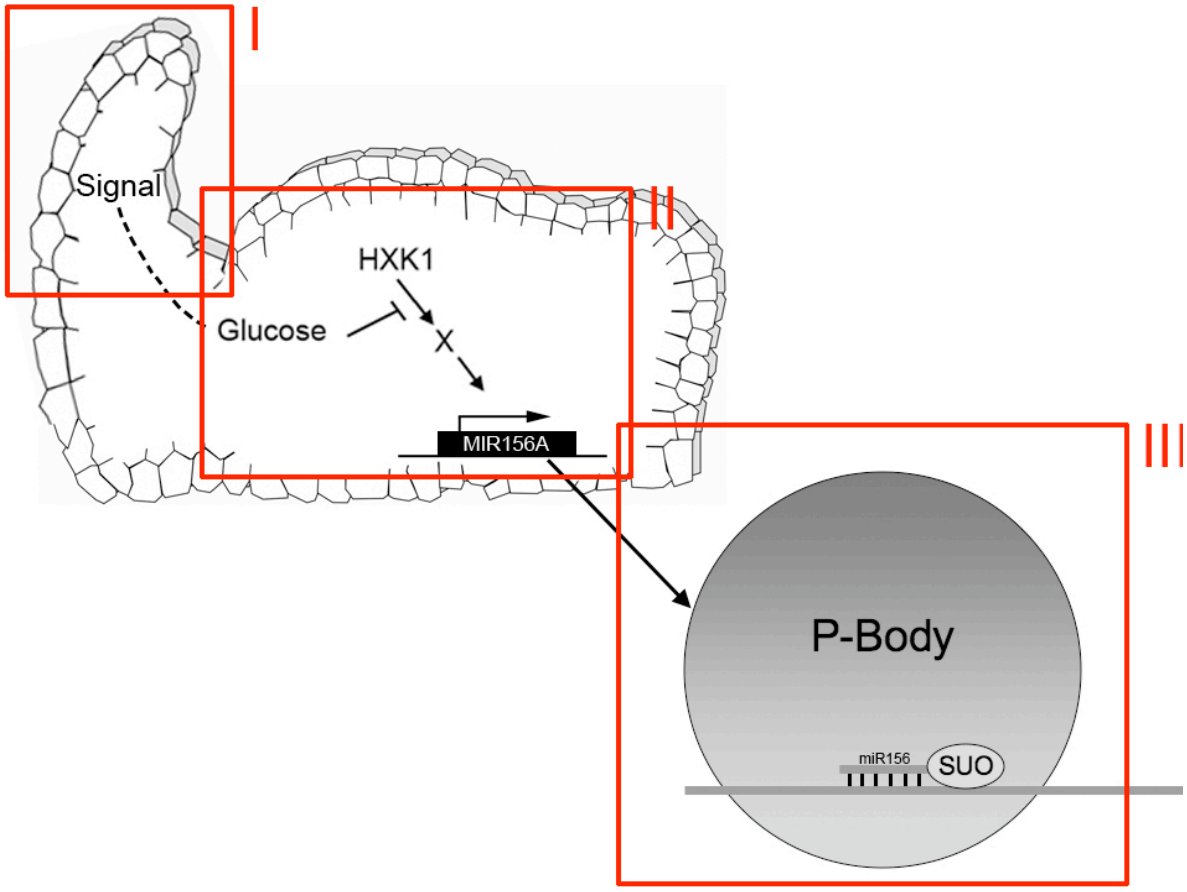


Figure 5.1: A summary of the regulation of miR156

A temporal decrease of miR156 is sufficient and necessary to trigger the phase change. An investigation for the source of signals promoting phase change reveals that roots and cotyledons are not the essential sources of such signals. Instead, a leaf-derived signal can promote the phase change by repressing the transcription of *MIR156* genes (I). Sugar was investigated as a potential leaf-derived signal in promoting the phase change. miR156 level is regulate by sugar in a HXK1-dependent manner. Mutants with defective photosynthesis or sugar signaling alter the timing of the phase change. These findings suggest that plants measure the timing of phase change by sensing the sugar level from leaves (II). On the other hand, the action of miR156 is also tightly regulated. Identified as a modifier of the miR156-SPL pathway, SUO is a component of the P-body and is specifically required for miRNA-mediated translational repression. The presence of GW motifs in SUO suggests that it is a partner of AGO proteins (III).

5.2.Future directions

Many questions remain to be explored in the field of phase change. Here, the discussion of possible future directions focuses on the regulation of miR156.

5.2.1. How does sugar-signaling pathway regulate miR156 expression?

Results in chapter III demonstrate that sugars regulate miR156 expression in an HXK1-dependent way. However, the detailed mechanism is still unknown. The activity of the full length pMIR156A::GUS reporter decreases upon glucose supply, which provides us an ideal tool to screen for factors that involved in this process. To identify the components required for the sugar-mediated repression of miR156. I propose to conduct a EMS screen in the *ch1*/*pMIR156A::LUC* background. *ch1* mutant has a reduced level of endogenous sugar, which makes it sensitive to exogenous sugar supply. Transgenic *ch1* plants carrying *pMIR156A::LUC* will be planted on sugar-deprived plates. A high level of luminescence is expected in such condition. At day 8, the seedling will be submerged in 100mM of glucose medium for 6 hours. Control plants will have reduced luminescence level after glucose treatment, while mutants with defective sugar-mediated repression of miR156 will show the same level of luminescence. The comparison is done with the same seedling before and after glucose treatment, which eliminates the mutants affecting carbohydrate availability instead of signaling.

Another interesting question related to this topic is whether sugar serves as a mobile signal from leaf to regulate the expression of *MIR156* genes in the meristem. To

address this question, I propose to use a FRET (fluorescence resonance energy transfer)-based glucose nanosensor to monitor the glucose level in meristem in real time. The response and efficiency of this glucose nanosensor has been test in leaf epidermis and root cells (Deuschle et al., 2006). In order to examine the correlation between sugar and miR156 level, the glucose concentration in meristem will be compared with the miR156 level reflected by a miR156 sensor (see *5.2.3*).

5.2.2. Investigate the role of chromatin remodeling in controlling MIR156 expression

Accumulating evidence implies that the regulation of chromatin structure is important for the temporal accumulation of miR156. A *pkl* (*pickle*) allele was isolated as a suppressor of *sqn* in our lab. *PKL* encodes a nuclear-localized chromatin-remodeling factor of the CHD3 subgroup. Preliminary results show that the temporal decrease of miR156 is delayed in *pkl* (Figure 5.2 A). As a consequence, miR172 accumulation is lower than that in wild type (Figure 5.2 B). In addition, an allele of *brm* (*brahma*), a SWI/SNF chromatin remodeling ATPase, was isolated in the screen for enhancers of eGFP-SPL3. The *brm* mutant has obvious precocious phenotypes such as serrated leaves and early onset of abaxial trichome (Figure 5.2 C, D). These results suggest that *MIR156* expression is regulated at the level of chromatin structure.

The role of chromatin remodeling in phase change has not been intensively investigated, which is partly due to the pleiotropic phenotypes caused by mutations in chromatin remodeling factors. Given that miR156 is a central regulator of phase change,

it is worthwhile to examine the miR156 expression in mutants of chromatin remodeling factors, such as *clf, emf*. In addition, it is necessary to analyze the temporal occupancy of histone modifications (e.g. H3K27me3) on the promoters of *MIR156* genes. If we can find a type of modification that is associated with the temporal expression of miR156, the next step is to see whether mutants with altered miR156 level affect such modification.

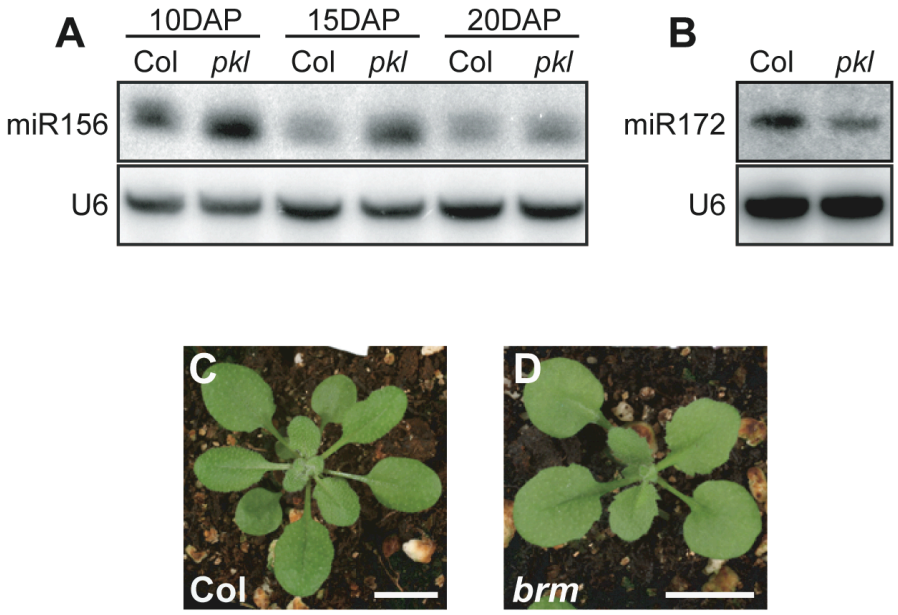


Figure 5.2: *pkl* and *brm* affect phase change.

A) *pkl* delays the temporal reduction of miR156. B) miR172 level decreases in *pkl*. C) and D) Compared to wild type Col-0 (C), *brm* mutant has a slow rate of leaf initiation and serrated leaf margin, which are typical phenotypes with reduced miR156 function. Scale bar in (C) and (D)=1cm.

5.2.3. Screen for mutants with delayed phase change

The current understanding of phase change largely relies on characterizing precocious mutants identified from genetic screens. However, a screen for mutants with delayed phase change has not yet been conducted. To identify factors that control the temporal expression of miR156, it is necessary to conduct a genetic screen for delayed phase change mutants. Previous screens for phase change mutants are based on heteroblastic phenotypes, such as the leaf shape and the trichome distribution. There are two major drawbacks using heteroblastic traits in a screen for delayed phase change. First, the appearance of abaxial trichome and the differences in leaf shape cannot be scored until plants are three-weeks old. Handling a large amount of adult Arabidopsis and flipping each leaf to score trichomes are very time-consuming and labor-intensive. Second, the discovery that the regulation of miR156 involves carbohydrate sensing suggests that mutants with delayed phase change may have pleiotropic defects, which makes phenotype-based screen difficult. To solve these problems, I generated two miR156-sensors for the endogenous miR156 level (Figure 5.3). In the *35S::eGFP-UTR$_{spl3}$* reporter, a constitutive 35S promoter drives a miR156-sensitive eGFP. The transgenic plants start to express eGFP in leaf 3 (Figure 5.3 A). PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT 1) is a putative MYB domain containing transcription factor involved in anthocyanin metabolism. In the *pPAP1::mPAP1-UTR$_{spl3}$* reporter, a modified PAP1 coding region is fused to the 3' UTR of *SPL3*, and is driven under its native promoter. As expected, the transgenic plants start to make purple leaves at leaf 3 (Figure 5.3 B). The expression of these reporters (eGFP and anthocyanin) fits the

temporal pattern of miR156. The sensitivity of these reporters to miR156 level is being tested. Once a stable and sensitive reporter line is obtained. We can use it to screen for mutants with late appearance of GFP signal or purple leaf, which indicates an extention of a high miR156 level.

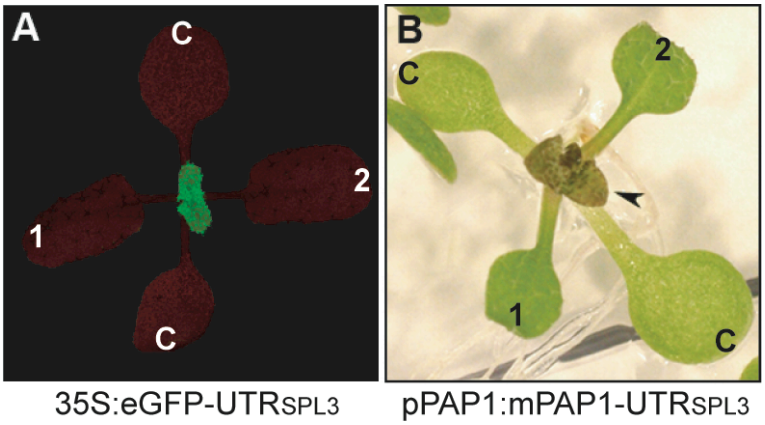


Figure 5.3: miR156 sensors show a temporal expression pattern

A) Transgenic plants carrying 35S:eGFP-UTR$_{SPL3}$ start to express GFP in leaf 3. No signal is detected in cotyledons and the first two leaves. B) In a low phosphate condition, anthocyanin is accumulated in the third leaf of pPAP1:mPAP1-UTR$_{SPL3}$. C: cotyledon; numbers indicate the position of the leaves. Arrowhead in B points to a purple leaf with anthocyanin accumulation.

6. MATERIAL AND METHODS

Growth Conditions

Seeds were sown on fertilized Fafard #2 soil (Fafard) and left at 4ºC for 2 days prior to being transferred to a growth chamber. The plant age was measured from the time the seeds were transferred to the growth chamber. The normal growth condition was under continuous fluorescent light (100 E/minute/m2; Sylvania VHO) at 22°C. The short day condition is 10 hrs light:14 hrs dark, 23º C under a 3:1 combination of cool white (F032/841/Eco, Sylvania) and wide spectrum (Gro Lite WS, Interlectric Corp.) fluorescent lights, at a light intensity of 200 μmol/m^2/sec. Glucose and sucrose response were examined on agar plates with ½ MS and different sugars in the short-day condition.

Genetic Stocks

All of the Arabidopsis genetic stocks used were in a Columbia background, unless otherwise specified. The enhancer trap E1735 was generated in our laboratory using a GAL4—UAS::GFP vector provided by Jim Haseloff (Haseloff, 1999). The UAS::DTA line (Laplaze et al., 2005) was also obtained from Jim Haseloff . This transgene was then transferred from C24 into Columbia by 5 rounds of backcrossing. An enhancer trap line that expresses GFP in hydathodes (E325) was used to examine the effect of defoliation on the hydathode number. *Ch1* was a gift from Robert Bassi; Ler, *gin2-1*, *HXK1*/*gin2-1,* and *S177A*/*gin2-1* seeds were from Brandon Moore. *gin2* (SALK_070739), *vha-b1* (SALK_028728), *rpt-5b* (SALK_069366), *suo-3* (SALK_074555), *suo-4* (SALK_020387), *suo-5* (SALK_060573), and *suo-6* (CS836050) were obtained from the

Arabidopis Biological Resource Center (Columbus, OH). *suo-2* was identified as a second-site mutation in a transgenic line. It is unlinked to the transgene.

Transgenic Plants

We first generated the p3300-Gateway-GUS+ plasmid. For this purpose, the GUS+ cassette in pCAMBIA3301-GUS+ was amplified using the forward primer 5'-(BamHI)-GGATCCATGGTAGATCTGAGGGTAAATTTCTAGTTTTTCTCC-3'

and the reverse primer 5'-(SacI)-GAGCTCCACTGATAGTTTAATTCCCGATCTAGTAACATAG -3'. This PCR product was cloned into the pCAMBIA 3300 vector using Sac I and BamHI restriction sites. The Gateway cassette was amplified from pEarleyGate 202 using the forward primer 5'-GCGAAGCTTAATTAAGCGCGGCGCGCCGGACACGCTCGAGATCACAAG -3' and the reverse primer 5'- GCCTAGGCACCACTTTGTACAAG-3'. The pCambia 3300 GUS+ plasmid was cut with BamHI, and the overhangs were blunted using a Klenow enzyme. The Gateway PCR product was then cloned into this plasmid, generating p3300-Gateway-GUS+.

To generate pSPL9::SPL9-GUS+ and pSPL9::rSPL9-GUS+, a 5.2 kb fragment containing the SPL9 promoter and coding region was amplified and cloned upstream of GUS+ in p3300-Gateway-GUS+ using the primers in Table 1. The miR156-resistant rSPL9 gene was produced by introducing silent mutations into the PCR primers that were used to amplify this gene. To generate pSPL3::rSPL3-GUS+, a 3.4 kb fragment containing the SPL3 promoter and the SPL3 coding region without the 3'-UTR (which

contains the miR156 target site) was amplified and introduced into p3300-Gateway-GUS+. To generate pSPL3::GUS+-SPL3, the GUS+ coding region was inserted into a 3.9 kb fragment containing the *SPL3* genomic region, between the SPL3 promoter and its start codon using PCR fusion. The whole sequence was amplified and cloned into p3300 between the EcoRI and BamHI sites. These constructs were generated by Conway SR.

To construct pSPL3::GFP-SPL3, a 2.9 kb genomic fragment upstream of the ATG and a 0.9 kb fragment downstream of the ATG of *SPL3* were cloned before and after eGFP, respectively, in the binary vector pCAMBIA3300 (CAMBIA) and transformed into Col-0 plants.

To generate 35S::SUO-eGFP, the coding region of AT3G48050 was amplified from BAC T17F15 with primers listed in Table 2. The PCR fragment was inserted into the Nco I site in pCAMBIA3301-eGFP. The plasmid was sequenced and transformed into agrobacteria GV3101. A floral dip was performed to transform Col-0 wild type and *suo-2*.

Phenotype analysis

Abaxial trichomes were scored with a stereomicroscope. For leaf shape analysis, fully expanded leaves were removed, attached to cardboard with double-sided tape and flattened with transparent tape, and then scanned in a digital scanner. Rips in the leaf blade produced during this process were filled in using Photoshop. The hydathode number was counted using the E325 enhancer trap line under an OLYMPUS MVX10 (OLYMPUS).

To measure drought resistance, Col-0 and *suo-2* were planted randomly in a 96-well tray. Watering was stopped upon bolting, and the number of dead and surviving plants with each genotype was counted two weeks later.

To test their sensitivity to ABA, Col-0 and *suo* seeds were planted on ½ MS plates without sucrose, containing different concentrations of ABA. The germination frequency was determined 6 days after moving the plates to a growth chamber.

Mutagenesis

Mutagenesis was performed according to (Hunter et al., 2006). Seeds were bulk harvested from groups of approximately 200 M1 plants. About 40 bulks of M2 progeny were screened for elevated GFP expression at 14 days after germination with a stereomicroscope. The candidate mutants from the same bulk with a similar phenotype were crossed for the complementation test. After the complementation test, the expression levels of miR156 and miR159 were examined in each candidate mutant.

Defoliation

The first two leaves of 8 to 12-day-old Arabidopsis seedlings grown in short days were removed using forceps. At the first time point, these leaves were about 1 mm long. A small wound on leaf 1 or leaf 2 was made at the same time on control plants. The gene expression was analyzed in the shoots (excluding cotyledons and the first two leaves) harvested 3 days after defoliation. Cotyledon(s) or the first leaf of *Nicotiana benthamiana* plants grown in long days were removed when the plants were 2 weeks old. Shoot apices with leaves of less than 1 cm long were harvested 3 days after manipulation. Shoot

apices from 3-week-old maize seedlings were dissected and cultured as described in (Orkwiszewski and Poethig, 2000). The first two leaves of *Acacia Mangium* were removed four weeks after germination. The shoot apices (excluding leaf 1 and leaf 2) were harvest 8 days after defoliation. The number pinnate leaves were counted three months later when the first phyllode expanded.

Small RNA northern

RNA blots were processed as described previously (Wu and Poethig, 2006). Briefly, plant tissues were homogenized in liquid nitrogen. Total RNAs from these tissues were extracted using a Trizol reagent. To isolate small RNAs, the total RNAs were incubated on ice with 500mM NaCl and 5% PEG8000 for 2 hours and centrifuged at 13,000 rpm for 10 minutes. Supernatants were collected and incubated with 1/10 volume 3M NaOAc and 2 volume of 95% ethanol at -20°C for 2 hours. Small RNAs were then precipitated by centrifuging at 13,000 rpm for 10 minutes. The concentration of small RNAs was quantified using a nanodrop spectrophotometer before being loaded on polyacrylamide gel.

Real-time PCR

The total RNAs were extracted using a Trizol reagent (Invitrogen) and cleaned with a Qiagen RNeasy mini kit (Qiagen). The clean RNAs were quantified and reverse transcribed into the first strand of cDNA using Invitrogen SuperScript™ II Reverse Transcriptase (Invitrigen). cDNAs were diluted and used as templates for real-time PCR.

The PCRs were performed in a Power SYBR Green PCR Master Mix (Applied Biosystems) using *ACTIN* or *eIF4* as a standard.

Western blot

Western blots were processed according to (Earley et al., 2010) and were incubated with anti-GFP (Sigma-G1544) or anti-CSD1/CSD2 (a gift of Dan Kliebenstein, U. C., Davis) at room temperature for 2 hours. The Col-0 and *suo* mutants used for the analysis of *CSD1/CSD2* expression were grown in Farfard #2 soil without added fertilizer.

GUS staining

To examine the consequences caused by defoliation, the fifth leaves were harvested from the control and defoliated plants 6 days after defoliation and stained with X-Gluc solution (Gold biotechnology) using the protocol described in (Senecoff et al., 1996). The incubation time for the rSPL3-GUS+ and rSPL9-GUS+ reporters was reduced to 1 hour to compensate for the high level of GUS activity in these lines.

To test the sugar response, the sixth leaves at a 5mm length were detached from SPL9-GUS+ and rSPL9-GUS+ reporter lines. These leaves were shaken for 10 minutes in different mediums and then kept at room temperature overnight. After the over-night incubation, the leaves were submerged into X-Gluc solution and evacuated. The leaves with X-Gluc solution were kept at 37°C incubator for 6 hours. Finally, the chlorophyll was washed off using 70% ethanol to obtain a white background.

Microscopy

The pSPL3::eGFP-SPL3 construct and the generation of transgenic plants containing this construct have been previously described (Yamaguchi et al., 2009). To generate the 35S::SUO-eGFP construct, the SUO coding region was inserted into the NcoI site in P3301-GUS, and the GUS sequence in P3301-GUS was replaced by eGFP at the NcoI and BstEII sites. The primers used to generate this construct are listed in Table 2. The DCP1::DCP1-CFP construct was a gift from Nam Hai Chua (Rockefeller U.). These constructs were transformed into *Agrobacterium tumefaciens* GV3101 and co-infiltrated into *Nicotiana benthamiana* leaves. Fluorescence was examined using a Leica confocal microscope.

Promoter truncation analysis

To generate the full length *MIR156A* reporter lines, 6kb of the *MIR156A* genomic sequence was cloned into a pCAMBIA3301 vector at BamH I and BstE II sites. Then, the stem-loop structure of *MIR156A* was replaced by GUS+. The full length *MIR156A* reporter was generated by Keith Earley. The truncated versions of the *MIR156A* reporter were generated by replacing the upstream or downstream regulatory sequence with truncated PCR products. More than 40 individual transgenic lines from each construct were analyzed, and transgenic lines harboring stable single insertions were selected for further analysis.

For promoter truncation analysis, 8-day old seedlings were harvested from plates with or without sucrose. The seedling were treated with 90% acetone on ice for 10 minutes, and then washed with water three times before staining them with X-Gluc.

Table 2 Primers and Oligos

For Genotyping	
gin2-1 (R)	ATTGGAGTGAGTGACTTCAACG
gin2-1(F)-PstI	AGATACTACTAAAGACGAGGAGCTG
gin2-1(F)-Afl III	GATACTACTAAAGACGAGGA**C**GTG
see149-F-EcoRI (suo-1)	GGTTGTAGCGGCAACAGACAAG
see149-R-EcoRI (suo-1)	AGCCACTCATCAAACACAGGGAAT
tm1-F (suo-2)	TCATTCTTATCGACCTAATGTG
tm1-R (suo-2)	GCTCATCACCAGCAACAAGTG
SALK_074555-F (suo-3)	CAAGCTGTTTGTACCTCTGTCAGTAC
SALK_074555-R (suo-3)	GCAGCAGCAGCAACAGTAATAGATGCACGAG
SALK_020387-F (suo-4)	TGTGAAGCTGCCGAATCGTG
SALK_020387-R (suo4)	GCTCAGTACTGACAGAGGTACA
SALK_060573-F (suo-5)	ACGTGGTCAAAACCGTCCGTC
SALK_060573-R (suo-5)	CCCTGAGAGAGAAAAGAGTTAC
CS836050-F (suo-6)	GGTTGTAGCGGCAACAGACAAG
CS836050-R (suo-6)	GCTCAGTACTGACAGAGGTACA
sqn-1.dCAP.BsII R	TCTGAGAGTAAATCAAGGTCAAA
sqn-1.dCAP.BsII F	GAAAGCCCAGCTGCCTTATCTTG
ago1-46.dCAP.Nhe.R	TGATGTCTCTGGCTCCATGTAGAAGCTAG
ago1-46.dCAP.Nhe.F	TGCAAGATGCACACGCTCAGTTTC
ago1-45.dCAP.Sph1.F	TGAGCCATGGTCTCGGATGTTTCA
ago1-45.dCAP.Sph1.R	GAGACTATGCCGAGTTCAGTCTCACGCATG
spl9-LP (SAIL_150_B05)	TGGTTCCTCCACTGAGTCATC
spl9-RP (SAIL_150_B05)	GCTCATTATGACCAGCGAGTC
spl15-LP (SALK_074426)	TGTTGGTGTCTGAAGTTGCTG
spl15-RP (SALK_074426)	AGGAAGCCAAAACCATAATGG

SALK_086029F(AT3G48060)	CAAGCTGTTCGCCCCACTCTCAGTAA
SALK_086029R(AT3G48060)	CAGCAACAGTAATGGATGCAGGAA
For pSPL3::eGFP-SPL3	
pSPL3::eGFP-SPL3-2	CAGCTCCTCGCCCTTGCTCACCATCTGCAAAATTCAACTCTCTC
pSPL3::eGFP-SPL3-3	GAGAGAGTTGAATTTTGCAGATGGTGAGCAAGGGCGAGGAGCTG
pSPL3::eGFP-SPL3-4	CTTTGCTTCTTCTCATACTCATCTTGTACAGCTCGTCCATGCCGAG
pSPL3::eGFP-SPL3-5	CTCGGCATGGACGAGCTGTACAAGATGAGTATGAGAAGAAGCAAAG
SPL3-G1	CCGGAATTCCTGTAAAGATAATTGTG
SPL3-G2	GGCCGGATCCGATTAGTCTTCCAATC
For 35S::SUO-eGFP	
SUO-GFP-F-NcoI	AACATGCCATGGATGCATGGGAGGGTTTGTGAGCG
SUO-GFP-R2-NcoI	AACATGCCATGGCTTGCCATGAGGACTGCCTATATC
For SUO mapping	
DSPM1	CTTATTTCAGTA AGAGTGTGGGGTTTTGG
DSPM5	CGGGATCCGACACTCTTTAATTAACTGACACTC
N106514-RP	AAGAGGCAGCCAAAACCTATC
N106514-LP	GCTTATGACAAGGCTGCAATC
F1P2(A)-F	TTTGGTTGGCCCGTAGATGTATCC
F1P2(A)-R	GCCAGATGCTCTGTTGGCATCTTT
M48120-R(XhoI)	TACTAGATTAACCACCTCGA
M48120-F(XhoI)	CCAATCGAGATTCTTAGAGCTC

M47950-F(Pvu II)	TCAACTGTTCATCGAACAGC
M47950-R(Pvu II)	CATCATTACCTCCTTTGTATC
M48030-F(Bgl II)	CTGATCTCATTTCACGAGAT
M48030-R(Bgl II)	TTCTGAATCTCATGATTCAAACG
MAT3G48010-R(ScaI)	TTTCGTCTCCGTTGTTAGTA
MAT3G48010-F(ScaI)	AACCAGATACTATTCGCATC
For RACE	
CUC2-R	TCAGTAGTTCCAAATACAGTCAAG
ARF8-R	CTAGAGATGGGTCGGGTTTTGCG
PHV-R	GACTCATAAAGAGGCCTGAGG
MYB65-R	AACTCTCTTTGGTTCCCAAACC
For RT-PCR	
ACT2-R	AACCCTCGTAGATTGGCACA
ACT2-F	GCACCCTGTTCTTCTTACCG
qMIR156A-F	CAAGAGAAACGCAAAGAAACTGACAG
qMIR156A-R	AAAGAGATCAGCACCGGAATCTGACAG
qMIR156B-F	GCTAGAAGAGGGAGAGATGGTGATTGAG
qMIR156B-R	GTGAGCACGCACACGCAAAGTTATAGAC
qMIR156C-F	AAGAGAAACGCATAGAAACTGACAG
qMIR156C-R	GGGACCGAATCGGAGCCGGAATCTGAC
qMIR156D-F	GGGAAGTTGTATAAAAGTTTTGTATATGG
qMIR156D-R	TGGTATGCAGAGACAGATAAGAAC
qMIR156F-F	GATGAAGCAAGTCAACTAAAGGAG
qMIR156F-R	GCAGGAGACAAGAAGAGAGTAAG
qMIR156H-F	GAAAGAGAGCACAACCTGGGATTAGC

qMIR156H-R	CGCAATGATGGTGGCAGAAGGAAAGAG
qSPL3-F	CTTAGCTGGACACAACGAGAGAAGGC
qSPL3-R	GAGAAACAGACAGAGACACAGAGGA
qSPL9-F	CAAGGTTCAGTTGGTGGAGGA
qSPL9-R	TGAAGAAGCTCGCCATGTATTG
qSPL13-F	CCAATCTCTTCTTCTCCAAACAGTACCAGAAGC
qSPL13-R	GAAGCAAATGAGGGACTGACGACG
miR159a-F	GGAGCTCTACTTCCATCGTCA
miR159a-R	CCACGTTCTCATCAAAACTTTC
miR166a-F	GACTCTGGCTCGCTCTATTCA
miR166a-R	TGGTCCGAAGACGCTAAAAC
miR167a-F	GAAGCTGCCAGCATGATCTA
miR167a-R	GGGTTTATAGAAGGGTGCGA
miR171a-F	CCGCGCCAATATCTCAGTA
miR171a-R	TGTCTCCATTTCAACACACACA
q-CSD2-F	CAACTAATGGATGTATCTCAACAGGACC
q-CSD2-R	GCCACGCCATCGGCATTGGCATTTATG
qCSD1-F	GCGAAAGGAGTTGCAGTTTTG
qCSD1-R	ACCATGAAGACCAGGCTTAAG
qSUO-F	GCGACTTCACAGTGGTCTCAAC
qSUO-R	CCACTCCGGTGAATTTAGGAC
MYB33(At5g06100)-F	TCGTCATCTCCTCCACACTCTG
MYB33(At5g06100)-R	CCTCGGATTTAGTTTGGGATAC
MYB65-F	GATGGTTCCTGATAGCCATACAGTTAC
MYB65-R	TAGGCATCAACAGAGTCAAGGAGATC
CUC2-F	GCACCAACACAACCGTCACAG
CUC2-R	GAATGAGTTAACGTCTAAGCCCAAGG

ARF8-F	AGATGTTTGCTATCGAAGGGTTGTTG
ARF8-R	CCATGGGTCATCACCAAGGAGAAG
eIF4-F	AAACTCAATGAAGTACTTGAGGGAC
eIF4-R	TCTCAAAACCATAAGCATAAATACCC
For MIR156A reporter	
P156a-5'-F1(EcoR I)	GAATTCGTTTGAGAATGTGTCTTGTAAAAGTGACAGATCC
P156a-5'-R1(Nco I)	CCATGGGTTTCTTTGCGTTTCTCTTGTCCC
P156a -5'-F2 (EcoR I)	AAGAATTCCAAAGGACACCATTATTCACTCTC
P156a -5'-F3 (EcoR I)	AAGAATTCCATTGCCATTTTTAGGTCTCTC
P156a-5'-F4 (EcoR I)	AAGAATTCCTCGATTTAGACAAAAACCCTAG
P156a-3'-F1(Pml I)	CACGTGGATTCCGGTGCTGATCTCTTTGGCC
p156a-3'-R1(BstE II)	GGTGACCGTTGTCTACTTTGTTTGATATGTGACGAC
P156a-3'-R2(BstE II)	AAGGTGACCGTGGCTAATTGGGTGATCACAGAC
P156a-3'-R3(BstE II)	AAGGTGACCCAAAAGTGGGAAGACATGACACATC
P156a-3'-R4(BstE II)	AAGGTGACCGAGTTTGTCGTTTGCGTTTAG
For small RNA northern	
miR165	GGGGGATGAAGCCTGGTCCGA
miR167	TAGATCATGCTGGCAGCTTCA
miR171	GATATTGGCGCGGCTCAATCA
miR398	AAGGGGTGACCTGAGAACACA
miR169	TCGGCAAGTCATCCTTGGCTG
miR161	CCCCGATGTAGTCACTTTCAA
miR164	TGCACGTGCCCTGCTTCTCCA
miR172	ATGCAGCATCATCAAGATTCT
miR156	GTGCTCACTCTCTTCTGTCA

miR159	TAGAGCTCCCTTCAATCCAAA
U6	AGGGGCCATGCTAATCTTCTC
tRNA-met	TCGAACTCTCGACCTCAGGAT
For AT3G48060 Expression	
RT-AT3G48060-F	CAAGCTGTTCGCCCCACTCTCAGTAA
RT-AT3G48060-R	CAGCAACAGTAATGGATGCAGGAA

7. REFERENCE

Allsopp, A. (1954). Juvenile stages of plants and the nutritional status of the shoot apex. Nature **173,** 1032-1035.

Allsopp, A. (1955). Ann. Botany (London) **19**.

Allsopp, A. (1965). Heteroblastic development in cormophytes. In Encyclopedia of Plant Physiology, W. Ruhland, ed (Berlin: Springer), pp. 1172-1221.

Allsopp, A. (1967a). Heteroblastic development in vascular plants. In Advances in Morphogenesis, ed. M Abercrombie, J Brachet **6,** 127-171.

Allsopp, A. (1967b). Heteroblastic development in vascular plants. Adv. Morphol. **6,** 127-171.

Amasino, R. (2010). Seasonal and developmental timing of flowering. Plant J **61,** 1001-1013.

Ambros, V. (2000). Control of developmental timing in Caenorhabditis elegans. Curr Opin Genet Dev **10,** 428-433.

Arazi, T., Talmor-Neiman, M., Stav, R., Riese, M., Huijser, P., and Baulcombe, D.C. (2005). Cloning and characterization of micro-RNAs from moss. Plant J **43,** 837-848.

Ashby, A. (1948). Studies on the morphogenesis of leaves I. An essay on leaf shape. New Phytol. **47,** 153-176.

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

Axtell, M.J., Snyder, J.A., and Bartel, D.P. (2007). Common functions for diverse small RNAs of land plants. Plant Cell **19,** 1750-1769.

Azevedo, J., Garcia, D., Pontier, D., Ohnesorge, S., Yu, A., Garcia, S., Braun, L., Bergdoll, M., Hakimi, M.A., Lagrange, T., and Voinnet, O. (2010). Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. Genes Dev **24,** 904-915.

Barakat, A., Wall, K., Leebens-Mack, J., Wang, Y.J., Carlson, J.E., and Depamphilis, C.W. (2007). Large-scale identification of microRNAs from a basal eudicot (Eschscholzia californica) and conservation in flowering plants. Plant J **51,** 991-1003.

Bassiri, A., Irish, E.E., and Poethig, R.S. (1992). Heterochronic Effects of Teopod-2 on the Growth and Photosensitivity of the Maize Shoot. Plant Cell **4,** 497-504.

Beauclair, L., Yu, A., and Bouche, N. (2010). microRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in Arabidopsis. Plant J **62,** 454-462.

Berardini, T.Z., Bollman, K., Sun, H., and Poethig, R.S. (2001). Regulation of vegetative phase change in Arabidopsis thaliana by cyclophilin 40. Science **291,** 2405-2407.

Bies-Etheve, N., Pontier, D., Lahmy, S., Picart, C., Vega, D., Cooke, R., and Lagrange, T. (2009). RNA-directed DNA methylation requires an AGO4-interacting member of the SPT5 elongation factor family. EMBO Rep **10,** 649-654.

Birkenbihl, R.P., Jach, G., Saedler, H., and Huijser, P. (2005). Functional dissection of the plant-specific SBP-domain: overlap of the DNA-binding and nuclear localization domains. J Mol Biol **352,** 585-596.

Bodson, M., and Outlaw, W.H. (1985). Elevation in the Sucrose Content of the Shoot Apical Meristem of Sinapis alba at Floral Evocation. Plant Physiol **79,** 420-424.

Bollman, K.M., Aukerman, M.J., Park, M.Y., Hunter, C., Berardini, T.Z., and Poethig, R.S. (2003). HASTY, the Arabidopsis ortholog of exportin 5/MSN5, regulates phase change and morphogenesis. Development **130,** 1493-1504.

Bouquin, T., Mattsson, O., Naested, H., Foster, R., and Mundy, J. (2003). The Arabidopsis lue1 mutant defines a katanin p60 ortholog involved in hormonal control of microtubule orientation during cell growth. J Cell Sci **116,** 791-801.

Brand, M.H., and Lineberger, R.D. (1992). In vitro rejuvenation of Betulaceae: morphological evaluation. Am. J. Bot. **79,** 618-625.

Brink, R.A. (1962). The quarterly review of biology: Phase change in higher plants and somatic cell heredity **37, NO. 1,** 1-22.

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L., and Voinnet, O. (2008). Widespread translational inhibition by plant miRNAs and siRNAs. Science **320,** 1185-1190.

Burk, D.H., and Ye, Z.H. (2002). Alteration of oriented deposition of cellulose microfibrils by mutation of a katanin-like microtubule-severing protein. Plant Cell **14,** 2145-2160.

Burk, D.H., Liu, B., Zhong, R., Morrison, W.H., and Ye, Z.H. (2001). A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. Plant Cell **13,** 807-827.

Callebaut, I., Courvalin, J.C., and Mornon, J.P. (1999). The BAH (bromo-adjacent homology) domain: a link between DNA methylation, replication and transcriptional regulation. FEBS Lett **446,** 189-193.

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

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

Chary, S.N., Hicks, G.R., Choi, Y.G., Carter, D., and Raikhel, N.V. (2008). Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in Arabidopsis. Plant Physiol **146,** 97-107.

Chen, X. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science **303,** 2022-2025.

Chien, J.C., and Sussex, I.M. (1996a). Differential regulation of trichome formation on the adaxial and abaxial leaf surfaces by Gibberellins and photoperiod in Arabidopsis thaliana (L) Heynh. PLANT PHYSIOL **111,** 1321-1328.

Chien, J.C., and Sussex, I.M. (1996b). Differential regulation of trichome formation on the adaxial and abaxial leaf surfaces by gibberellins and photoperiod in Arabidopsis thaliana (L.) Heynh. Plant Physiol **111,** 1321-1328.

Cho, Y.H., Yoo, S.D., and Sheen, J. (2006). Regulatory functions of nuclear hexokinase1 complex in glucose signaling. Cell **127,** 579-589.

Chuck, G., Cigan, A.M., Saeteurn, K., and Hake, S. (2007). The heterochronic maize mutant Corngrass1 results from overexpression of a tandem microRNA. Nat Genet **39,** 544-549.

Corbesier, L., Bernier, G., and Perilleux, C. (2002). C : N ratio increases in the phloem sap during floral transition of the long-day plants Sinapis alba and Arabidopsis thaliana. Plant and Cell Physiology **43,** 684-688.

Coruzzi, G.M., and Zhou, L. (2001). Carbon and nitrogen sensing and signaling in plants: emerging 'matrix effects'. Curr Opin Plant Biol **4,** 247-253.

Csorba, T., Lozsa, R., Hutvagner, G., and Burgyan, J. (2010). Polerovirus protein P0 prevents the assembly of small RNA-containing RISC complexes and leads to degradation of ARGONAUTE1. Plant J **62,** 463-472.

Day, M.E., Greenwood, M.S., and Diaz-Sala, C. (2002). Age- and size-related trends in woody plant shoot development: regulatory pathways and evidence for genetic control. Tree Physiology **22,** 507-513.

Deyholos, M.K., Cavaness, G.F., Hall, B., King, E., Punwani, J., Van Norman, J., and Sieburth, L.E. (2003). VARICOSE, a WD-domain protein, is required for leaf blade development. Development **130,** 6577-6588.

Deuschle, K., Chaudhuri, B., Okumoto, S., Lager, I., Lalonde, S., and Frommer, W.B. (2006). Rapid metabolism of glucose detected with FRET glucose nanosensors in epidermal cells and intact roots of Arabidopsis RNA-silencing mutants. Plant Cell **18,** 2314-2325.

Ding, L., Spencer, A., Morita, K., and Han, M. (2005). The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. Mol Cell **19,** 437-447.

Ding, X.C., and Grosshans, H. (2009). Repression of C. elegans microRNA targets at the initiation level of translation requires GW182 proteins. EMBO J **28,** 213-222.

Doorenbos, J. (1954). "Rejuvenation" of Hedera helix in graft combinations. Proc. Koninkl. Nederl. Akademie van Wetenschappen Ser. C **57,** 99-102.

Dudley, M., and Poethig, R.S. (1993). The heterochronic Teopod1 and Teopod2 mutations of maize are expressed non-cell-autonomously. Genetics **133,** 389-399.

Earley, K., Smith, M., Weber, R., Gregory, B., and Poethig, R. (2010). An endogenous F-box protein regulates ARGONAUTE1 in Arabidopsis thaliana. Silence **1,** 15.

El-Shami, M., Pontier, D., Lahmy, S., Braun, L., Picart, C., Vega, D., Hakimi, M.A., Jacobsen, S.E., Cooke, R., and Lagrange, T. (2007). Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. Genes Dev **21,** 2539-2544.

Espineda, C.E., Linford, A.S., Devine, D., and Brusslan, J.A. (1999). The AtCAO gene, encoding chlorophyll a oxygenase, is required for chlorophyll b synthesis in Arabidopsis thaliana. Proc Natl Acad Sci U S A **96,** 10507-10511.

Eulalio, A., Huntzinger, E., and Izaurralde, E. (2008). Getting to the root of miRNA-mediated gene silencing. Cell **132,** 9-14.

Eulalio, A., Tritschler, F., and Izaurralde, E. (2009a). The GW182 protein family in animal cells: new insights into domains required for miRNA-mediated gene silencing. RNA **15,** 1433-1442.

Eulalio, A., Helms, S., Fritzsch, C., Fauser, M., and Izaurralde, E. (2009b). A C-terminal silencing domain in GW182 is essential for miRNA function. RNA **15,** 1067-1077.

Evans, M.M., and Poethig, R.S. (1995). Gibberellins promote vegetative phase change and reproductive maturity in maize. Plant Physiol **108,** 475-487.

Evans, M.M., Passas, H.J., and Poethig, R.S. (1994). Heterochronic effects of glossy15 mutations on epidermal cell identity in maize. Development **120,** 1971-1981.

Fabian, M.R., Sundermeier, T.R., and Sonenberg, N. (2010). Understanding how miRNAs post-transcriptionally regulate gene expression. Prog Mol Subcell Biol **50,** 1-20.

Fornara, F., de Montaigu, A., and Coupland, G. (2010). SnapShot: Control of flowering in Arabidopsis. Cell **141,** 550, 550 e551-552.

Fortanier, E.J., and Jonkers, H. (1976). Juvenility and maturity of plants as influenced by their ontogenetical and physiological ageing. Acta Hort. **56,** 37-44.

Foster, T., Hay, A., Johnston, R., and Hake, S. (2004). The establishment of axial patterning in the maize leaf. Development **131,** 3921-3929.

Fraga, M.F., Rodriguez, R., and Canal, M.J. (2003). Reinvigoration of Pinus radiata is associated with partial recovery of juvenile-like polyamine concentrations. Tree Physiology **23,** 205-209.

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

Frank, H., and Renner, O. (1956). Über Verjüngung bei Hedera helix L. Planta **47,** 105-114.

Franks, T.M., and Lykke-Andersen, J. (2008). The control of mRNA decapping and P-body formation. Mol Cell **32,** 605-615.

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

Gibson, S.I. (2005). Control of plant development and gene expression by sugar signaling. Current Opinion in Plant Biology **8,** 93-102.

Giner, A., Lakatos, L., Garcia-Chapa, M., Lopez-Moya, J.J., and Burgyan, J. (2010). Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. PLoS Pathog **6,** e1000996.

Goebel, K. (1900). Organography of plants Part I. General organography. (English translation by I. B. Balfour). (Oxford: Clarendon Press).

Goeres, D.C., Van Norman, J.M., Zhang, W., Fauver, N.A., Spencer, M.L., and Sieburth, L.E. (2007). Components of the Arabidopsis mRNA decapping complex are required for early seedling development. Plant Cell **19,** 1549-1564.

Gomez, L.D., Baud, S., and Graham, I.A. (2005). The role of trehalose-6-phosphate synthase in Arabidopsis embryo development. Biochem Soc Trans **33,** 280-282.

Gomez, L.D., Baud, S., Gilday, A., Li, Y., and Graham, I.A. (2006). Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. Plant J **46,** 69-84.

Gomez, L.D., Gilday, A., Feil, R., Lunn, J.E., and Graham, I.A. (2010). AtTPS1-mediated trehalose 6-phosphate synthesis is essential for embryogenic and vegetative growth and responsiveness to ABA in germinating seeds and stomatal guard cells. Plant J **64,** 1-13.

Gould, K.S. (1993). Leaf Heteroblasty in Pseudopanax-Crassifolius - Functional-Significance of Leaf Morphology and Anatomy. Ann Bot-London **71,** 61-70.

Guo, A.Y., Zhu, Q.H., Gu, X., Ge, S., Yang, J., and Luo, J. (2008). Genome-wide identification and evolutionary analysis of the plant specific SBP-box transcription factor family. Gene **418,** 1-8.

Hanson, J., Johannesson, H., and Engstrom, P. (2001). Sugar-dependent alterations in cotyledon and leaf development in transgenic plants expressing the HDZhdip gene ATHB13. Plant Molecular Biology **45,** 247-262.

Haseloff, J. (1999). GFP variants for multispectral imaging of living cells. Methods Cell Biol **58,** 139-151.

He, X.J., Hsu, Y.F., Zhu, S., Wierzbicki, A.T., Pontes, O., Pikaard, C.S., Liu, H.L., Wang, C.S., Jin, H., and Zhu, J.K. (2009). An effector of RNA-directed DNA

methylation in arabidopsis is an ARGONAUTE 4- and RNA-binding protein. Cell **137,** 498-508.

Heyer, A.G., Raap, M., Schroeer, B., Marty, B., and Willmitzer, L. (2004). Cell wall invertase expression at the apical meristem alters floral, architectural, and reproductive traits in Arabidopsis thaliana. Plant J **39,** 161-169.

Hsieh, L.C., Lin, S.I., Shih, A.C., Chen, J.W., Lin, W.Y., Tseng, C.Y., Li, W.H., and Chiou, T.J. (2009). Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. Plant Physiol **151,** 2120-2132.

Huang, L.C., Lius, S., Huang, B.L., Murashige, T., Mahdi, E.F.M., and Vangundy, R. (1992). Rejuvenation of Sequoia sempervirens by repeated grafting of shoot tips onto juvenile rootstocks invitro - model for phase reversal of trees. Plant Physiology **98,** 166-173.

Hunter, C., Sun, H., and Poethig, R.S. (2003). The Arabidopsis heterochronic gene ZIPPY is an ARGONAUTE family member. Curr Biol **13,** 1734-1739.

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

Husen, A., and Pal, M. (2003). Effect of serial bud grafting and etiolation on rejuvenation and rooting cuttings of mature trees of Tectona grandis Linn. f. Silvae Genetica **52,** 84-88.

IAN M. SUSSEX, M.E.C. (1960). A study of the effect of extrernally supplied sucrose on the morphology of excised fern leaves in vitro. phytomorphology **10,** 87-99.

Irish, E.E., and Karlen, S. (1998). Restoration of juvenility in maize shoots by meristem culture. Intl. J. Plant Sci. **159,** 695-701.

Jang, J.C., Leon, P., Zhou, L., and Sheen, J. (1997). Hexokinase as a sugar sensor in higher plants. Plant Cell **9,** 5-19.

Jones, C.S. (1995). Does shade prolong juvenile development? A morphological analysis of leaf shape changes in Cucurbita argyrosperma Subsp. Sororia (Cucurbitaceae). Amer. J. Bot. **82,** 346-359.

Jones, C.S. (1999). An Essay on Juvenility, Phase Change, and Heteroblasty in Seed Plants. Int J Plant Sci **160,** S105-S111.

Jones, L., Keining, T., Eamens, A., and Vaistij, F.E. (2006). Virus-induced gene silencing of argonaute genes in Nicotiana benthamiana demonstrates that extensive systemic silencing requires Argonaute1-like and Argonaute4-like genes. Plant Physiol **141,** 598-606.

Jung, J.H., Seo, Y.H., Seo, P.J., Reyes, J.L., Yun, J., Chua, N.H., and Park, C.M. (2007). The GIGANTEA-regulated microRNA172 mediates photoperiodic flowering independent of CONSTANS in Arabidopsis. Plant Cell **19,** 2736-2748.

Karlowski, W.M., Zielezinski, A., Carrere, J., Pontier, D., Lagrange, T., and Cooke, R. (2010). Genome-wide computational identification of WG/GW Argonaute-binding proteins in Arabidopsis. Nucleic Acids Res **38,** 4231-4245.

Kasschau, K.D., Xie, Z., Allen, E., Llave, C., Chapman, E.J., Krizan, K.A., and Carrington, J.C. (2003). P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. Dev Cell **4,** 205-217.

Kerstetter, R.A., and Poethig, R.S. (1998). The specification of leaf identity during shoot development. Annu Rev Cell Dev Biol **14,** 373-398.

Kerstetter, R.A., Bollman, K., Taylor, R.A., Bomblies, K., and Poethig, R.S. (2001). KANADI regulates organ polarity in Arabidopsis. Nature **411,** 706-709.

Klein, J., Saedler, H., and Huijser, P. (1996). A new family of DNA binding proteins includes putative transcriptional regulators of the Antirrhinum majus floral meristem identity gene SQUAMOSA. Mol Gen Genet **250,** 7-16.

Koch, K. (2004). Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr Opin Plant Biol **7,** 235-246.

Komeda, Y. (2004). Genetic regulation of time to flower in Arabidopsis thaliana. Annu Rev Plant Biol **55,** 521-535.

Lanet, E., Delannoy, E., Sormani, R., Floris, M., Brodersen, P., Crete, P., Voinnet, O., and Robaglia, C. (2009). Biochemical evidence for translational repression by Arabidopsis microRNAs. Plant Cell **21,** 1762-1768.

Laplaze, L., Parizot, B., Baker, A., Ricaud, L., Martiniere, A., Auguy, F., Franche, C., Nussaume, L., Bogusz, D., and Haseloff, J. (2005). GAL4-GFP enhancer trap lines for genetic manipulation of lateral root development in Arabidopsis thaliana. J Exp Bot **56,** 2433-2442.

Lauter, N., Kampani, A., Carlson, S., Goebel, M., and Moose, S.P. (2005). microRNA172 down-regulates glossy15 to promote vegetative phase change in maize. Proc Natl Acad Sci U S A **102,** 9412-9417.

Lee, D.W., and Richards, J.H. (1991). Heteroblastic development in vines. In The Biology of Vines, F.E. Putz and H.A. Mooney, eds (New York, NY: Cambridge University Press), pp. 205-243.

Lee, H., Yoo, S.J., Lee, J.H., Kim, W., Yoo, S.K., Fitzgerald, H., Carrington, J.C., and Ahn, J.H. (2010). Genetic framework for flowering-time regulation by ambient temperature-responsive miRNAs in Arabidopsis. Nucleic Acids Res **38,** 3081-3093.

Lejeune, P., Bernier, G., and Kinet, J.M. (1991). Sucrose Levels in Leaf Exudate as a Function of Floral Induction in the Long Day Plant Sinapis-Alba. Plant Physiol Bioch **29,** 153-157.

Lejeune, P., Bernier, G., Requier, M.C., and Kinet, J.M. (1993). Sucrose Increase during Floral Induction in the Phloem Sap Collected at the Apical Part of the Shoot of the Long-Day Plant Sinapis-Alba L. Planta **190,** 71-74.

Levy, Y.Y., and Dean, C. (1998). Control of flowering time. Curr Opin Plant Biol **1,** 49-54.

Liu, Q., Yao, X., Pi, L., Wang, H., Cui, X., and Huang, H. (2009). The ARGONAUTE10 gene modulates shoot apical meristem maintenance and leaf polarity establishment by repressing miR165/166 in Arabidopsis. Plant J **58,** 27-40.

Liu, X., and Gorovsky, M.A. (1993). Mapping the 5' and 3' ends of Tetrahymena thermophila mRNAs using RNA ligase mediated amplification of cDNA ends (RLM-RACE). Nucleic Acids Res **21,** 4954-4960.

Llave, C., Xie, Z., Kasschau, K.D., and Carrington, J.C. (2002). Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science **297,** 2053-2056.

Lynn, K., Fernandez, A., Aida, M., Sedbrook, J., Tasaka, M., Masson, P., and Barton, M.K. (1999). The PINHEAD/ZWILLE gene acts pleiotropically in Arabidopsis development and has overlapping functions with the ARGONAUTE1 gene. Development **126,** 469-481.

Mallory, A.C., Hinze, A., Tucker, M.R., Bouche, N., Gasciolli, V., Elmayan, T., Lauressergues, D., Jauvion, V., Vaucheret, H., and Laux, T. (2009). Redundant and specific roles of the ARGONAUTE proteins AGO1 and ZLL in development and small RNA-directed gene silencing. PLoS Genet **5,** e1000646.

McConnell, J.R., and Barton, M.K. (1995). Effect of mutations in the pinhead gene of Arabidopsis on the formation of shoot apical meristems. Dev. Genet. **16,** 358-366.

Milyaeva, E.L., and Komarova, E.N. (1996). Changes in the sugar content in stem apices of the short-day plant Perilla nankinensis at floral transition. Russ J Plant Physl+ **43,** 149-154.

Milyaeva, E.L., Komarova, E.N., and Kochankov, V.G. (1996). Floral evocation and the content of carbohydrates in stem apices of Rudbeckia bicolor at floral transition in complete darkness. Russ J Plant Physl+ **43,** 259-262.

Miura, K., Ikeda, M., Matsubara, A., Song, X.J., Ito, M., Asano, K., Matsuoka, M., Kitano, H., and Ashikari, M. (2010). OsSPL14 promotes panicle branching and higher grain productivity in rice. Nat Genet **42,** 545-549.

Monteuuis, O., and Bon, M.C. (1989). Rejuvenation of a 100 yr old giant sequoia (Sequoiadendron giganteum Buchholz) through in vitro meristem culture. Annales Des Sciences Forestieres **46,** S183-S186.

Moon, H.K., Park, S.Y., Kim, Y.W., and Kim, S.H. (2008). Somatic embryogenesis and plantlet production using rejuvenated tissues from serial grafting of a mature Kalopanax septemlobus tree. In Vitro Cell Dev-Pl **44,** 119-127.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Liu, Y.X., Hwang, I., Jones, T., and Sheen, J. (2003). Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science **300,** 332-336.

Moose, S.P., and Sisco, P.H. (1994a). Glossy15 Controls the Epidermal Juvenile-to-Adult Phase Transition in Maize. Plant Cell **6,** 1343-1355.

Moose, S.P., and Sisco, P.H. (1994b). *Glossy15* controls the epidermal juvenile-to-adult phase transition in maize. Plant Cell **6,** 1343-1355.

Moreno, M.A., Harper, L.C., Krueger, R.W., Dellaporta, S.L., and Freeling, M. (1997). liguleless1 encodes a nuclear-localized protein required for induction of ligules and auricles during maize leaf organogenesis. Genes Dev **11,** 616-628.

Moose, S.P., and Sisco, P.H. (1996). Glossy15, an APETALA2-like gene from maize that regulates leaf epidermal cell identity. Genes Dev **10,** 3018-3027.

Mouradov, A., Cremer, F., and Coupland, G. (2002). Control of flowering time: interacting pathways as a basis for diversity. Plant Cell **14 Suppl,** S111-130.

Moussian, B., Schoof, H., Haecker, A., Jurgens, G., and Laux, T. (1998). Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during Arabidopsis embryogenesis. EMBO J **17,** 1799-1809.

Mullins, M.G., Nair, Y., and Sampet, P. (1979). Rejuvenation in vitro - induction of juvenile characters in an adult clone of Vitis vinifera L. Annals of Botany **44,** 623-&.

Njoku, E. (1956a). Studies in the morphogenesis of leaves XI. The effect of light intensity on leaf shape in Ipomea caerulea. New Phytol. **55,** 91-110.

Njoku, E. (1956b). The effect of defoliation on leaf shape in *Ipomoea caerulea*. New Phytol **55,** 213-228.

Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., and Ohme-Takagi, M. (2001). Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell **13,** 1959-1968.

Ohto, M., Onai, K., Furukawa, Y., Aoki, E., Araki, T., and Nakamura, K. (2001). Effects of sugar on vegetative development and floral transition in arabidopsis. Plant Physiology **127,** 252-261.

Orkwiszewski, J.A., and Poethig, R.S. (2000). Phase identity of the maize leaf is determined after leaf initiation. Proc Natl Acad Sci U S A **97,** 10631-10636.

Oster, U., Tanaka, R., Tanaka, A., and Rudiger, W. (2000). Cloning and functional expression of the gene encoding the key enzyme for chlorophyll b biosynthesis (CAO) from Arabidopsis thaliana. Plant J **21,** 305-310.

Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature **425,** 257-263.

Park, M.Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H., and Poethig, R.S. (2005). Nuclear processing and export of microRNAs in Arabidopsis. Proc Natl Acad Sci U S A **102,** 3691-3696.

Park, W., Li, J., Song, R., Messing, J., and Chen, X. (2002). CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Curr Biol **12,** 1484-1495.

Pasquinelli, A.E., and Ruvkun, G. (2002). Control of developmental timing by micrornas and their targets. Annu Rev Cell Dev Biol **18,** 495-513.

Perilleux, C., and Bernier, G. (1997). Leaf carbohydrate status in Lolium temulentum during the induction of flowering. New Phytol **135,** 59-66.

Pien, S., Wyrzykowska, J., and Fleming, A.J. (2001). Novel marker genes for early leaf development indicate spatial regulation of carbohydrate metabolism within the apical meristem. Plant J **25,** 663-674.

Poethig, R.S. (1988a). Heterochronic mutations affecting shoot development in maize. Genetics **119,** 959-973.

Poethig, R.S. (1988b). Heterochronic mutations affecting shoot development in maize. Genetics **119,** 959-973.

Poethig, R.S. (1990). Phase change and the regulation of shoot morphogenesis in plants. Science **250,** 923-930.

Poethig, R.S. (2003). Phase change and the regulation of developmental timing in plants. Science **301,** 334-336.

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

Poethig, R.S. (2010). The past, present, and future of vegetative phase change. Plant Physiol **154,** 541-544.

Poethig, S. (1988c). A Non-Cell-Autonomous Mutation Regulating Juvenility in Maize. Nature **336,** 82-83.

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. (2002). MicroRNAs in plants. Genes Dev **16,** 1616-1626.

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

Riese, M., Hohmann, S., Saedler, H., Munster, T., and Huijser, P. (2007). Comparative analysis of the SBP-box gene families in P. patens and seed plants. Gene **401,** 28-37.

Riese, M., Zobell, O., Saedler, H., and Huijser, P. (2008). SBP-domain transcription factors as possible effectors of cryptochrome-mediated blue light signalling in the moss Physcomitrella patens. Planta **227,** 505-515.

Roldan, M., Gomez-Mena, C., Ruiz-Garcia, L., Salinas, J., and Martinez-Zapater, J.M. (1999). Sucrose availability on the aerial part of the plant promotes morphogenesis and flowering of Arabidopsis in the dark. Plant J **20,** 581-590.

Rolland, F., Moore, B., and Sheen, J. (2002). Sugar sensing and signaling in plants. Plant Cell **14 Suppl,** S185-205.

Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol **57,** 675-709.

Ronemus, M., Vaughn, M.W., and Martienssen, R.A. (2006). MicroRNA-targeted and small interfering RNA-mediated mRNA degradation is regulated by argonaute, dicer, and RNA-dependent RNA polymerase in Arabidopsis. Plant Cell **18,** 1559-1574.

Schaffalitzky de Muckadell, M. (1954). Juvenile stages in woody plants. Physiol. Plant. **7,** 782-796.

Schauer, S.E., Jacobsen, S.E., Meinke, D.W., and Ray, A. (2002). DICER-LIKE1: blind men and elephants in Arabidopsis development. Trends Plant Sci **7,** 487-491.

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

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

Senecoff, J.F., McKinney, E.C., and Meagher, R.B. (1996). De novo purine synthesis in Arabidopsis thaliana. II. The PUR7 gene encoding 5'-phosphoribosyl-4-(N-succinocarboxamide)-5-aminoimidazole synthetase is expressed in rapidly dividing tissues. Plant Physiol **112,** 905-917.

Shikata, M., Koyama, T., Mitsuda, N., and Ohme-Takagi, M. (2009). Arabidopsis SBP-box genes SPL10, SPL11 and SPL2 control morphological change in association with shoot maturation in the reproductive phase. Plant Cell Physiol **50,** 2133-2145.

Smith, M.R., Willmann, M.R., Wu, G., Berardini, T.Z., Moller, B., Weijers, D., and Poethig, R.S. (2009). Cyclophilin 40 is required for microRNA activity in Arabidopsis. Proc Natl Acad Sci U S A **106,** 5424-5429.

Stoutemyer, V.T., and Britt, O.K. (1961). Effect of temperature and grafting on vegetative growth of Algerian ivy. Nature **189,** 854-855.

Strable, J., Borsuk, L., Nettleton, D., Schnable, P.S., and Irish, E.E. (2008). Microarray analysis of vegetative phase change in maize. Plant J **56,** 1045-1057.

Sunkar, R., Kapoor, A., and Zhu, J.K. (2006). Posttranscriptional Induction of Two Cu/Zn Superoxide Dismutase Genes in Arabidopsis Is Mediated by Downregulation of miR398 and Important for Oxidative Stress Tolerance. Plant Cell **18,** 2051-2065.

Sussex, M.E.C. (1960). A study of the effect of extrernally supplied sucrose on the morphology of excised fern leaves in vitro. phytomorphology **10,** 87-99.

Talmor-Neiman, M., Stav, R., Frank, W., Voss, B., and Arazi, T. (2006). Novel micro-RNAs and intermediates of micro-RNA biogenesis from moss. Plant J **47,** 25-37.

Telfer, A., Bollman, K.M., and Poethig, R.S. (1997). Phase change and the regulation of trichome distribution in Arabidopsis thaliana. Development **124,** 645-654.

Tsai, C.H., Miller, A., Spalding, M., and Rodermel, S. (1997). Source Strength Regulates an Early Phase Transition of Tobacco Shoot Morphogenesis. Plant Physiol **115,** 907-914.

Usami, T., Horiguchi, G., Yano, S., and Tsukaya, H. (2009). The more and smaller cells mutants of Arabidopsis thaliana identify novel roles for SQUAMOSA

PROMOTER BINDING PROTEIN-LIKE genes in the control of heteroblasty. Development **136,** 955-964.

van Dijken, A.J., Schluepmann, H., and Smeekens, S.C. (2004). Arabidopsis trehalose-6-phosphate synthase 1 is essential for normal vegetative growth and transition to flowering. Plant Physiol **135,** 969-977.

Vaucheret, H., Mallory, A.C., and Bartel, D.P. (2006). AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1. Mol Cell **22,** 129-136.

Vaucheret, H., Vazquez, F., Crete, P., and Bartel, D.P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Genes Dev **18,** 1187-1197.

Vazquez, F., Gasciolli, V., Crete, P., and Vaucheret, H. (2004). The Nuclear dsRNA Binding Protein HYL1 Is Required for MicroRNA Accumulation and Plant Development, but Not Posttranscriptional Transgene Silencing. Curr Biol **14,** 346-351.

Voinnet, O. (2009). Origin, biogenesis, and activity of plant microRNAs. Cell **136,** 669-687.

Wang, C., Ying, S., Huang, H., Li, K., Wu, P., and Shou, H. (2009a). Involvement of OsSPX1 in phosphate homeostasis in rice. Plant J **57,** 895-904.

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

Wang, J.W., Schwab, R., Czech, B., Mica, E., and Weigel, D. (2008). Dual effects of miR156-targeted SPL genes and CYP78A5/KLUH on plastochron length and organ size in Arabidopsis thaliana. Plant Cell **20,** 1231-1243.

Wang, J.W., Park, M.Y., Wang, L.J., Koo, Y., Chen, X.Y., Weigel, D., and Poethig, R.S. (2011). MiRNA Control of Vegetative Phase Change in Trees. PLoS Genet **7,** e1002012.

Wareing, P. (1959). Problems of juvenility and flowering in trees. Bot. J. Linn. Soc. Lond. **56,** 282-289.

Webb, M., Jouannic, S., Foreman, J., Linstead, P., and Dolan, L. (2002). Cell specification in the Arabidopsis root epidermis requires the activity of ECTOPIC ROOT HAIR 3--a katanin-p60 protein. Development **129,** 123-131.

Wiltshire, R.J.E., Potts, B.M., and Reid, J.B. (1991). A paedomorphocline in Eucalyptus - natural variation in the E. risdonii E. tenuiramis complex. Australian Journal of Botany **39,** 545-566.

Wong, C.E., Singh, M.B., and Bhalla, P.L. (2009). Molecular processes underlying the floral transition in the soybean shoot apical meristem. Plant Journal **57,** 832-845.

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

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

Xie, Z., Allen, E., Fahlgren, N., Calamar, A., Givan, S.A., and Carrington, J.C. (2005). Expression of Arabidopsis MIRNA genes. Plant Physiol **138,** 2145-2154.

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

Xu, J., and Chua, N.H. Processing bodies and plant development. Curr Opin Plant Biol.

Xu, J., Yang, J.Y., Niu, Q.W., and Chua, N.H. (2006). Arabidopsis DCP2, DCP1, and VARICOSE form a decapping complex required for postembryonic development. Plant Cell **18,** 3386-3398.

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

Yu, N., Cai, W.J., Wang, S., Shan, C.M., Wang, L.J., and Chen, X.Y. (2010). Temporal control of trichome distribution by microRNA156-targeted SPL genes in Arabidopsis thaliana. Plant Cell **22,** 2322-2335.

Zalenski, V. (1904). Cited in Maximov, N.A (1929). "The Plant in Relation to Water.". Allen & Unwin **London**.

Zhang, X., Yuan, Y.R., Pei, Y., Lin, S.S., Tuschl, T., Patel, D.J., and Chua, N.H. (2006). Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. Genes Dev **20,** 3255-3268.

Zhang, X., Zou, Z., Zhang, J., Zhang, Y., Han, Q., Hu, T., Xu, X., Liu, H., Li, H., and Ye, Z. (2011). Over-expression of sly-miR156a in tomato results in multiple

vegetative and reproductive trait alterations and partial phenocopy of the sft mutant. FEBS Lett **585,** 435-439.

Zimmerman, R.H., Hackett, W.P., and Pharis, R.P. (1985). Hormonal aspects of phase change and precocious flowering. In Encycl. Plant Physiol., New Series, R.P. Pharis and D.M. Reid, eds (Berlin; New York: Springer-Verlag), pp. 79-115.

8. APPENDIX

8.1. Four categories of *see* mutants

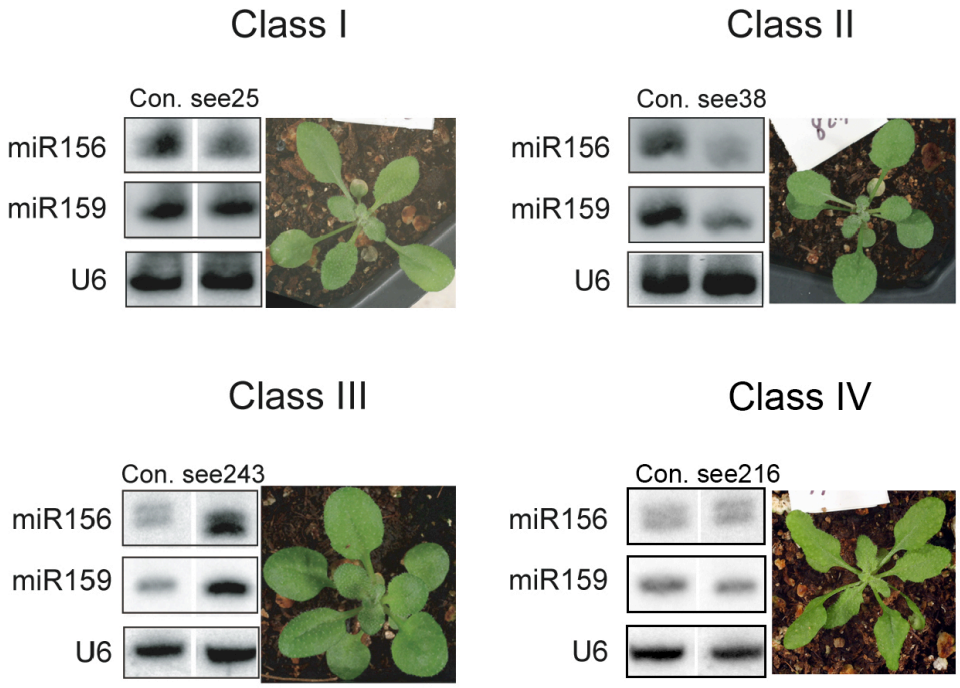


Figure 8.1 Three categories of see mutant.

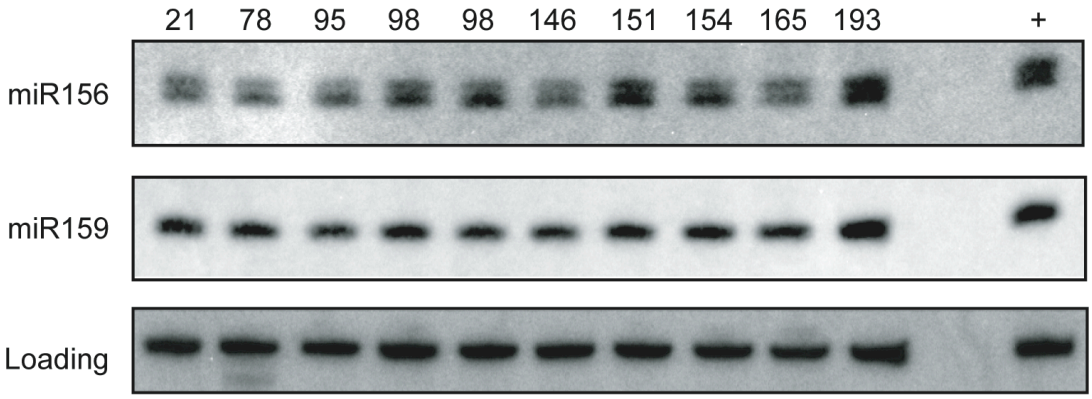
The class I mutants are with reduced miR156 and normal miR159 (e.g. *see25*); the class II mutants are with reduced miR156 and miR159 (e.g. *see38*); the class III mutants are with elevated level of miR156 and miR159 (e.g. *see243*); the class IV mutants do not change either miR156 or miR159 level (e.g. *see216*).

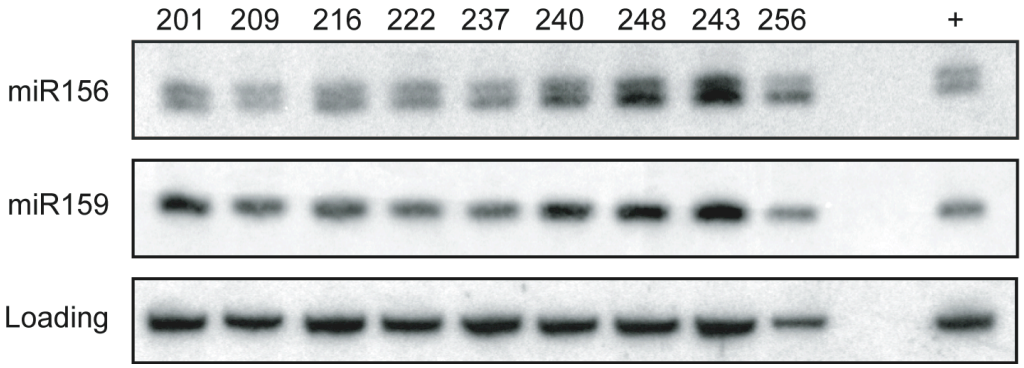
8.2. A summary of the phenotypes of *see* mutants

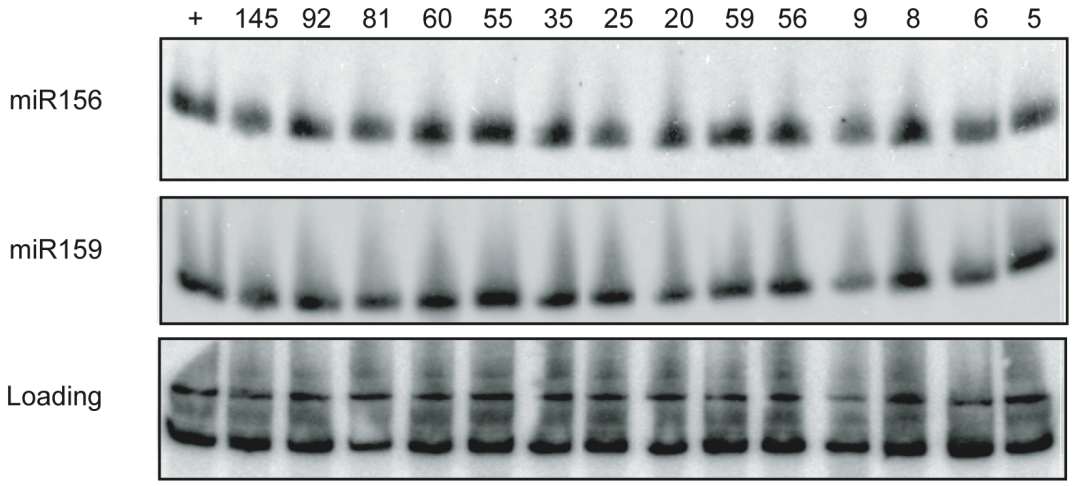
	Phenotype	No. of Juv.	miR156	miR159	Stock Number	Class
Control	N/A	7.25			4882	
see104	N/A	4.83	↓	—	3425	I
see124	weak ago1-like	4.25	↓	—	3736	I
see126	ago1-like	3.50			3661	
see135	similar as see124	6.60	↓	—	3733	I
see146	early flowering	5.50	↓	↓	3888	II
see154	weak sqn-like	3.67	↓	—	3858	I (*brm*)
see159	pin-like	5.80			3884	
see165	hen1-like	9.25	—	—	3833	IV
see18	se-like	7.67			3508	
see193	sqn-like	3.80	—	—	3934	IV
see201	weak sqn-like	3.50	↓	—	3933	I (*brm*)
see209	weak sqn-like	3.60	—	—	3932	IV
see21	N/A	6.20	↓	—	3547	I
see213	hst-phenotype	3.50			3931	
see216	early flowering	6.00	—	—	3814	IV
see229	leaf shape	6.25			3160	
see237	early flowering, early trichome	6.00	—	—	3416	IV
see240	early trichome, leaf shape	5.60	↑	↑	3150	III

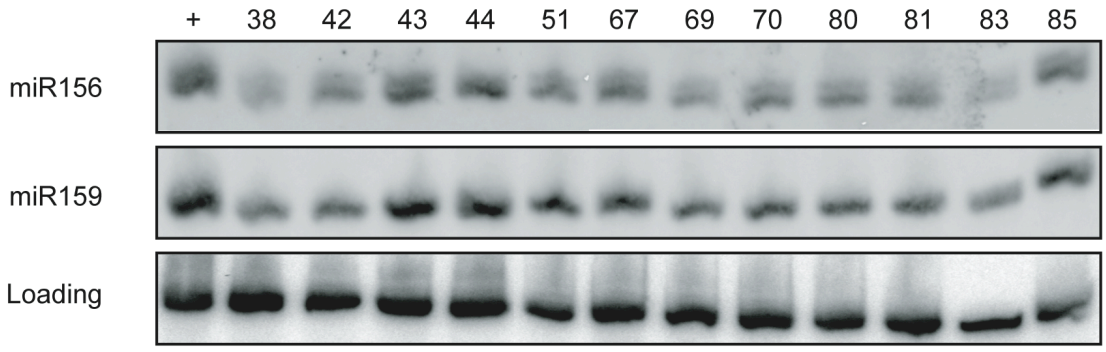
see241	early flowering, early trichome	7.00			3151	
see242	leaf shape	7.75			3152	
see243	leaf shape	4.25	↑	↑	3153	III
see244	leaf shape	6.00			3154	
see246	early trichome, leaf shape	6.75			2498	
see248	early trichome	5.20	↑	↑	3158	III
see25	SAM defect(minor),early trichome in SD	5.25	↓	—	3513	I
see38	more serration	6.25	↓	↓	3428	II
see42	N/A	8.00	↓	↓	3427	II
see43	N/A	8.00	—	—	3423	IV
see5	N/A	6.00	↓	—	3423	I
see51	N/A	6.50	↓	—	3433	I
see56	N/A	5.75	—	—	3434	IV
see6	N/A	8.00	↓	↓	3422	II
see69	slow rate of leaf initiation, pale green	5.20	↓	↓	3432	II
see78	elongated leaf, long hypocotyle	5.25	↓	—	3574	I
see8	N/A	7.00	—	—	3424	IV
see95	N/A	5.00	↓	↓	3435	II
see96	N/A	7.00	↓	—	3512	I
see98	long hypocotyle	5.67	—	—	3437	IV

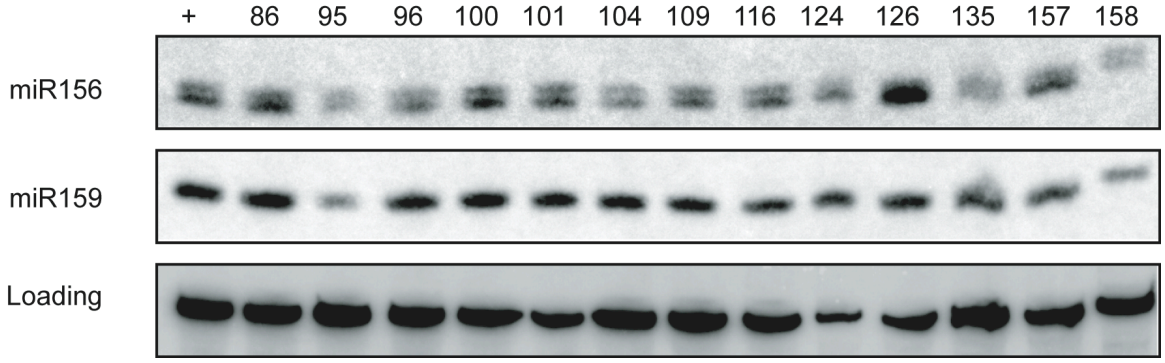
8.3. Original northern blots for *see* mutants






+ 145 92 81 60 55 35 25 20 59 56 9 8 6 5
miR156
miR159
Loading


+ 38 42 43 44 51 67 69 70 80 81 83 85
miR156
miR159
Loading


+ 86 95 96 100 101 104 109 116 124 126 135 157 158
miR156
miR159
Loading