ABSTRACT

HE, LUYAN. Spatial and Temporal Redistribution of MicroRNAs Associated with Maize Polysomes. (Under the direction of Dr. Heike Sederoff).

MicroRNAs (miRNAs) are sequence-specific regulatory elements of eukaryotic genomes. In plants, miRNAs typically assume the length of 20 to 22 nucleotides (nt), and act by base-pairing to silence the cognate mRNA target by either endonucleolytic cleavage or translational repression. Unlike in animals, where the base-pairing between miRNAs and targets is limited to a core 7-bp region within the miRNA:target hybrid, plant miRNAs and their targets exhibit extensive sequence complementarities. Therefore, two distinct modes of action have been proposed for animal and plant miRNAs. Animal miRNAs regulate gene expression via translational repression and/or destabilization of target mRNAs. In contrast, plant miRNAs predominantly silence gene expression by directing the target cleavage. This paradigm, however, has been challenged recently by several lines of evidence supporting miRNA-mediated translational repression as a widespread mechanism in plants.

In our project, we sought to investigate the roles of miRNAs as translational repressors during seed development and germination. We hypothesized that one of the many measures that plant miRNAs employ to control translation was to functionally interfere with the translational machinery. Therefore, we performed a Next-Gen sequencing study and subsequent RT-qPCR verification to identify miRNAs that were preferentially associated with polysomes in maize endosperm. Our data suggested that some miRNAs might bear intrinsic preference to associate with the translational machinery, while other miRNAs remained ribosome-free. In addition, our Next-Gen study revealed that some conserved

miRNAs in maize endosperm might have acquired new regulatory roles that were specific to maize endosperm. Furthermore, we sought to profile miRNA expression in maize embryos and endosperm from a broader spectrum of developmental stages. Our data suggested that (1) miRNAs were robustly involved in modulating gene expression to accommodate various developmental and physiological conditions and (2) some of these miRNA-imposed regulations may occur co-translationally. To gain a mechanistic insight into the miRNA-mediated translational control in plants, we performed immunoprecipitation studies using a transgenic Arabidopsis line overexpressing the FLAG-tagged ribosomal protein RPL18. We found that indeed miRNAs (with AGO1 protein) were associated with polysomes *in vivo*, and the extent of this interaction varied at different developmental stages. Interestingly, by surveying various protein phosphorylation databases, we found a plant AGO1-specific phosphorylation site near the AGO1 catalytic domain. Based on our data and previous findings, we propose a model where the phosphorylated AGO1 protein interacts with polysomes to repress the protein production from target mRNAs.

Spatial and Temporal Redistribution of MicroRNAs Associated with Maize Polysomes

by
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DEDICATION

I dedicate my dissertation to my grandparents Mr. Dianyuan He and Mrs. Xianling Liao, who dedicated their lives to the study of soil sciences in southern China, and who truly believe in the power of science to make the world a better place.

BIOGRAPHY

Luyan He was born in Changsha, China, in the year of 1985. She completed her Bachelor of Science degree in Biological Sciences from China Agricultural University in 2007. During her junior year in college she worked in the lab of Dr. De Ye as an undergraduate research assistant and for the first time, savored the joy of thinking like a real scientist. Not long after that she went to the Temasek Life Sciences Laboratory in Singapore to complete her final year project under the direction of Dr. Kanura Sampath. In the summer of 2007, she joined the department of plant biology at NC State University to pursue her doctorate degree. Since then, with the guidance and encouragement from her advisor Dr. Heike Winter Sederoff, she dived into the world of small RNAs.

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LIST OF ABBREVIATIONS

3’-UTRs	3’-untranslated regions
5’ RACE	5’ Rapid Amplification of cDNA Ends
ABA	Abscisic Acid
ABF	Auxin Signaling F-box
AGO	Argonaute
AI	After Imbibition
AP2	Apetala2
APS	ATP Sulferylase
ARE	AU-rich Element
ARF	Auxin Response Factor
CBC	Cap-biding Complex
CBP80	Cap-binding Protein 80
CCS1	Copper Chaperone for Superoxide Dismutase 1
CMT3	Chromomethylases 2
CSD1/2	Copper Superoxide Dismutases 1/2
CUC2	Cup-shaped Cotyledon 2
CyP40	Cyclophin 40
DAI	Day After Imbibition
DAP	Day After Pollination
D-body	Dicing Body
DCL1	Dicer-like 1

DCP	Decapping Enzyme
DDH	Aspartate-Aspartate-Histidine
DDL	Dawdle
DGCR8	DiGeorge Critical Region 8
DME	Demeter
DRM2	DNA (cytosine-5)-methyltransferase 2
dsRBD	dsRNA Binding Domain
dsRNA	Double-stranded RNA
ER	Endoplasmic Reticulum
FUS3	Fusca3
GA	Gibberellic Acid
GW protein	Glycine-tryptophan-rich Protein
HEN1	Hua Enhancer 1
hsiRNA	Heterochromatic siRNA
HSP90	Heat Shock Protein 90
HST	Hasty
HYL	Hyponastic Leaves 1
Imp8	Importin 8
IRES	Internal Ribosome Entry Site
KTN	Katanin
LEC2	Leafy-Cotyledon2
LSU	Large Subunit

miRNA	microRNA
NAC	No Apical Meristem; ATF1/2 and CUP-Shaped Cotyledon
nasiRNA	Natural antisense siRNA
ncRNA	Non-coding RNA
P body	Processing Body
PAPB	Poly-A Binding Protein
PAZ	Piwi-Argonaue-Zwille
PCD	Programmed Cell Death
PHB	Phabulosa
PHV	Phavoluta
PIWI	P-element Induced Wimpy Testis
PKC	Protein kinase C
POL II	RNA Polymerase II
POL IV	RNA Polymerase IV
PPIase	Peptidylprolyl Isomerase
pre-miRNA	Stem-loop miRNA Precursor
pri-miRNA	Primary RNA Precursor
PTGS	Post-transcriptional Gene Silencing
PTGS	Post-transcriptional Gene Silencing
RACK1	Receptor for Activated Kinase C
RISC	RNA-induced Silencing Complex
RLD 1/2	Rolled Leaf 1/2

RNP	Ribonucleoprotein
ROS	Reactive Oxygen Species
S/N	Supernatant
SCF Complex	Skp, Cullin, F-box Containing Complex
SCL6	Scarecrow-like 6
SDN1	Small RNA Degrading Nuclease-1
SE	Serrate
siRNA	Short Interfering RNA
snoRNA	Small Nucleolar RNA
snRNA	Small nuclear RNA
SOC1	Suppressor of Overexpression of Constans1
SPL	SQUAMOSA-promoter Binding-like
sRNA	Short RNA
SSU	Small Subunit
tasiRNA	Trans-acting siRNA
TCP	Teosinte Branched 1, Cycloidea, and PCF Family
TIR1	Transport Inhibitor Response1
TOE	Target of EAT
TRBP	TAR RNA-binding protein
TT1	Transparent Testa 1
TU	Transcription Unit
VCS	Varicose

Chapter 1

Literature Review

Overview

The control of mRNA turnover is an important component of the regulatory network of gene expression as the steady-state level of mRNA is determined by the rate of both synthesis and decay. In fact, one of the special features of mRNAs is their rapid turnover rate. Consequently, changes in the mRNA decay rate of specific genes provide a cell with flexibility to meet various physiological needs. Generally, in eukaryotes, mRNA stability is regulated by:

- Modifications at the 5' and 3' ends of mRNAs, which prevent exonucleases from attacking from either end of the mRNAs
- Nonsense-mediated RNA decay: A nonsense mutation in the coding region triggers a surveillance system that degrades the mRNA.
- Specific sequence elements within the 3' untranslated region (such as the AU-Rich Elements), whose roles are involved in either stabilizing or destabilizing the mRNA.
- Translational arrest of mRNAs in cytoplasmic foci such as P-bodies and stress granules.
- Non-coding RNA-mediated mRNA silencing.

The nomenclature of non-coding RNA (ncRNA) commonly refers to RNA that does not encode a protein. Although it is generally assumed that most genetic information is carried out by various protein functionalities, emerging evidence suggests that the majority of the genomes of higher eukaryotes are actually transcribed into ncRNAs (reviewed in Mattick and Makunin, 2006). Many ncRNAs are spliced and processed into smaller segments, among

which the most extensively studied groups are microRNAs (miRNAs) and short interfering RNAs (siRNAs; reviewed in Mattick and Makunin, 2006).

The initial findings in metazoans and plants that a certain group of ~22 nucleotide-long small RNA molecules were key post-transcriptional regulators of gene expression have since revolutionized our comprehension of the multitude of gene regulatory pathways (Napoli et al., 1990; van der Krol et al., 1990; Lee et al., 1993). Over the last decade, small RNA molecules have emerged to be critical regulators in the expression and function of eukaryotic genomes. Among these ncRNAs, siRNAs and miRNAs typically assume the length of 18 to 22 nt, and act in a broad range of eukaryotic species to regulate endogenous genes and defend the genome from being invaded by foreign genomic information (reviewed in Carthew and Sontheimer, 2009). Although siRNAs and miRNAs are structurally similar, they differ in that (1) miRNAs arise from precursors with stem-loop structures with incomplete double-strand character, while siRNAs generally are found to be excised from long and fully complementary double-stranded RNAs (dsRNAs); (2) miRNAs are endogenously and purposefully expressed products of an organism's own genome, while siRNAs can be derived from various places of origins such as centromeres, transposons, viruses and other repetitive regions; (3) miRNAs predominantly control gene expression at a post-transcriptional level, while siRNAs can contribute to genetic control from several aspects, from directing chromatin remodeling to silencing foreign or viral genes (reviewed in Carthew and Sontheimer, 2009). Many excellent reviews can be found elaborating on the biogenesis of plant and animal small RNAs, as well as the employed mechanisms for target selection

and function (Vazquez et al., 2004b; Cannell et al., 2008; Wu and Belasco, 2008; Kim et al., 2009; Krol et al., 2010a). In this chapter, I aimed to emphasize our current knowledge of miRNAs and their regulatory pathways, with a special interest in their interwoven roles with other mechanisms involved in RNA quality surveillance, RNA stability and translational control.

Plant miRNA biogenesis and regulatory pathways

Plant miRNA biogenesis

During the course of evolution, seed plants have adopted several different pathways that generate endogenous small RNAs of specific sizes. Based on their origins, small RNAs can be categorized into 4 distinct groups (Vazquez, 2006): miRNAs, heterochromatic siRNAs (hsiRNAs), trans-acting siRNAs (tasiRNAs), and natural antisense siRNAs (nasiRNAs). As mentioned earlier, the miRNA pathway generates 18-22 nt miRNAs from stem-loop precursors. The 24-nt hsiRNA derives from transposons and repeats, while the 21-nt or 24-nt nasiRNAs arise from dsRNAs precursors formed by two naturally paired transcripts (Willmann et al., 2011b). The production of the 21-nt tasiRNAs is triggered in a strictly phased manner by the activity of a special group of miRNAs and the associated protein factors. In plants, small RNAs regulate gene silencing at both transcriptional and post-transcriptional levels (reviewed in Voinnet, 2009; Henderson and Jacobsen, 2007). While hsiRNAs mainly direct chromatin remodeling and gene methylation in collaboration with RNA polymerase IV, V (POL IV, POL V) and other remodeling factors, miRNAs and

tasiRNAs regulate target genes by base-pairing and subsequently silence them by endonucleolytic cleavage or translational repression. Generally, plant RNA silencing events occur in five consensus steps: (1) induction by dsRNAs; (2) processing of dsRNAs into 18-24 nt sRNAs; (3) 3'-O-methylation of sRNAs; (4) incorporation of sRNAs into effector complexes; (5) association of effector complexes with partially or fully complementary target RNAs or DNAs. Several reviews have elucidated the biogenesis pathways and functions of plant small RNAs (Wassenegger, 2005; Zhang, 2006; Vaucheret, 2006; Naqvi et al., 2012). Here I have reviewed our current knowledge of plant miRNAs and mechanisms underlying the plant miRNA-mediated gene regulation.

In Figure 1.1, the biogenesis pathway of plant miRNAs is illustrated. Plant miRNAs are expressed under the influence of their own promoters. Similar to protein-coding genes, genes encoding miRNAs are transcribed by RNA polymerase II (POL II) to generate primary miRNA (pri-miRNA) transcripts, and this transcription is controlled by various transcription factors (reviewed in Bartel, 2004; Naqvi et al., 2012; Voinnet, 2009). Pri-miRNAs bear characteristic features of 5'cap, 3'-polyA and intron splicing (reviewed in Bartel 2004; Xie et al., 2005), and their lengths range from few hundred to several thousand bases (Zhang et al., 2009; Naqvi et al., 2012). The fold-back structure contained within pri-miRNAs is recognized by the RNA binding protein HYL1 (Hyponastic Leaves 1) and the zinc-finger protein Serrate (SE), which interact with Dicer-like 1 (DCL1) in nuclear processing centers called D-bodies (Kurihara et al., 2006; Lobbes et al., 2006; Fang and Spector, 2007; Song et al., 2007; Liu et al., 2012). While DCL1 or SE null mutants are embryonic-lethal, weak

alleles of *se* give rise to phenotypes resembling those caused by mutations in CBP80 and CBP20, which encode subunits of the nuclear cap-binding complex (CBC; Gregory et al., 2008; Laubinger et al., 2008). Like *se* mutants, *cbp80* and *cbp20* mutants accumulate more pri-miRNAs and fewer mature miRNAs. Conversely, weak *se* mutants exhibit the general mRNA splicing defects typical of *cbp80* and *cbp20* mutants (Gregory et al., 2008; Laubinger et al., 2008). Therefore, unlike HYL1 and DCL1, which function specifically in miRNA biogenesis, SE and CBC are involved in both miRNA processing and mRNA splicing pathways.

Similar to HYL1, DAWDLE (DDL) is also a nuclear RNA-binding protein. However, while HYL1 acts to facilitate the accurate processing of pri-miRNAs, DDL presumably functions in stabilizing pri-miRNAs, as pri-miRNAs accumulate poorly in *ddl* mutants despite unchanged transcription of miRNA genes (Yu et al., 2008). The nuclear processing of miRNAs occurs at two steps. (1) The endonucleolytic activity of DCL1 (with HYL1, SE, and CBC) releases the stem-loop region from the parent pri-miRNAs. The stem-loop structure is called pre-miRNA, and similar to pri-miRNAs, pre-miRNAs are also highly variable in their sizes (Zhang et al., 2006c). (2) Pre-miRNAs are further cleaved by DCL1 alone, to generate miR:miR* duplexes. One strand of this duplex is called the guide strand, which later will be bound by Argonaute1 (AGO1) protein, while the other strand, namely the passenger strand or miRNA*, is thought to undergo degradation (reviewed in Bartel, 2004; Naqvi et al., 2012; Voinnet, 2009). While still in the nucleus, miR:miR* duplexes are stabilized by the S-adenosyl methionine-dependent methyltransferase Hua Enhancer 1 (HEN1), which

methylates all miRNAs and siRNAs in plants (Li et al., 2005; Yu et al., 2005; Yang et al., 2006c). Methyl groups deposited on the 3' terminal nucleotides of each strand of the miR:miR* duplex have been shown to prevent the uridylation and subsequent degradation of plant small RNAs (Li et al., 2005; Zhao et al., 2012). A nuclear shuttle protein, HASTY1 (HST), has been demonstrated to facilitate the export of miRNA duplexes to the cytoplasm (Park et al., 2005). However, *hst* mutants show decreased accumulation of only some miRNAs in both nuclear and cytoplasmic fractions, which suggests the presence of an HST-independent miRNA export mechanism in plants (Park et al., 2005). Recently, another RNA-binding protein, TOUCH, has been shown to be involved in plant miRNA biogenesis, possibly by contributing to the recruitment of pri-miRNAs to the DCL1 processing complex (Ren et al., 2012).

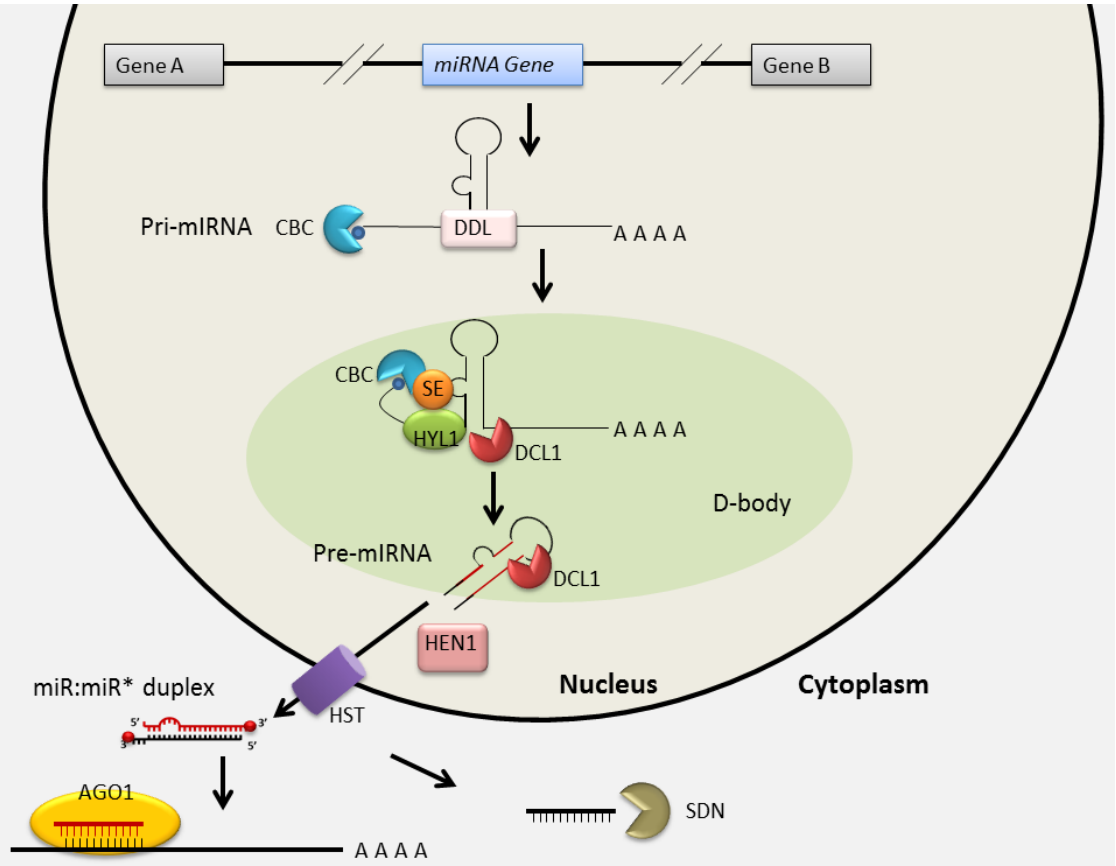

Figure 1.1 Plant miRNA biogenesis pathway.

Plant pri-miRNAs are transcribed by RNA POL II from intergenic regions. The RNA-binding protein DDL presumably stabilizes pri-miRNAs during their conversion to stem-loop pre-miRNAs in nuclear processing centers named D-bodies. This conversion also requires the coordinated action and physical interaction of the C2H2-zinc finger protein SE, the dsRNA-binding protein HYL1, DCL1, and the nuclear cap-binding complex (CBC). Pre-miRNAs, or miR:miR* duplexes are then exported to the cytoplasm via the action of the plant exportin 5 ortholog HASTY and maybe other unknown factors. Before nuclear export, miR:miR* duplexes excised from pre-miRNAs are methylated by HEN1 at 3'ends, a reaction that prevents them from being degraded by SDN. The mature miRNA strand is then recruited by the AGO1 protein to carry out the silencing reactions while the passenger strand (miR*) would eventually be degraded, possibly via the activity of SDN.

CBC: Cap-binding complex; DDL: Dawdle; SE:Serrate ; HYL Hyponastic leaves 1; DCL1: Dicer-like 1; HEN1: Hua Enhancer 1;HST: HASTY; AGO1: Argonaute 1; SDN: Small RNA degrading nuclease; D-body: Dicing body

In the cytoplasm, the miR:miR* duplex recruits a crucial miRNA effector protein termed AGO1 (Vaucheret et al., 2004; Vaucheret, 2006, 2008). It is proposed that the binding of AGO1 and possibly other accessory proteins (together referred to as the RNA-induced silencing complex, RISC) leads to the unwinding of miR:miR* duplexes to separate the guide strand (miR) from passenger strand (miR*). Generally, it is believed that the 5' thermodynamic stability of the two strands determines the selection of miRNA and miRNA* (Schirle and MacRae, 2012), with the one with lower thermostability being selected as the "guide strand" to direct target silencing (Tomari et al., 2004b; Tomari et al., 2004a; Gredell et al., 2010). In mammalian cells, correct RISC assembly is dependent on Dicer and the transactivating response (TAR) RNA-binding protein (Chendrimada et al., 2005; Gregory et al., 2005; Gredell et al., 2010; Carmel et al., 2012). In plants, HYL1 has been implicated in directing guide strand selection from the miR:miR* duplex (Eamens et al., 2009). HYL1 contains two dsRNA binding domains (dsRBDs) shared by the TAR RNA-binding protein (TRBP) in humans and the R2D2 protein in Drosophila (Eamens et al., 2009). Eamens et al. reported that reminiscent of the functions of TRBP and R2D2 in animals, in Arabidopsis, HYL1 was responsible for strand selection, possibly via thermodynamically orientating the miR:miR* duplex onto AGO1 for passenger degradation (Eamens et al., 2009).

Notably, miRNA biogenesis is different in plants and animals as (1) more than 50% of animal miRNAs can be clustered by their close proximity to neighboring miRNAs, and these clustered miRNAs arise from a single miRNA polycistronic transcription unit (TU; Lee et al., 2002) ; (2) animal miRNAs can be located in both non-coding transcripts and protein-coding

transcripts (reviewed in Kim et al., 2009); (3) in animals, pri-miRNAs are trimmed in the nucleus to release the stem-loop pre-miRNAs from the rest of the transcript by the activity of a complex comprised of an RNase III-like enzyme called Drosha and the dsRNA-binding protein DGCR8 (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004); (4) in animals, the stem-loop pre-miRNAs are exported to the cytoplasm by Exportin5, whereas in plants, it is believed that pre-miRNAs are processed in the nucleus into miR:miR* duplexes before entering the cytoplasm (Yi et al., 2003; Lund et al., 2004); (5) in the cytoplasm, animal pre-miRNAs are cleaved by Dicer, releasing the miR:miR* duplex (Bernstein et al., 2001; Hutvagner et al., 2001); (6) some animal dsRNA-binding proteins, such as HIV trans-activator RNA-binding protein (TRBP), act in concert with Dicer to direct the Dicer complex to AGO2 for gene silencing (Chendrimada et al., 2005; Haase et al., 2005); (7) in contrast to plant miRNAs, animal miRNAs are usually not methylated (reviewed in Aravin et al., 2007); (8) in contrast to other organisms which contain one (human, mouse, fission yeast and *C. elegans*) or two (Drosophila) Dicer enzymes, the Arabidopsis genome contains 4 DCL paralogs (Schauer et al., 2002). This difference might reflect the functional diversity of plant small RNAs.

Argonaute proteins: at the core of RNA silencing

Structural studies of Argonaute proteins (AGOs) in archeal (Song et al., 2004; Rivas et al., 2005), eubacterial (Yuan et al., 2005; Wang et al., 2008b; Wang et al., 2008a; Wang et al., 2009a) and human (Elkayam et al., 2012) systems have provided critical insights to understand miRNA-mediated target repression. Structurally, AGOs are characterized by 3

major functional domains: PAZ (Piwi-Argonaue-Zwille), MID (Middle), and PIWI (P-element induced wimpy testis; Bohmert et al., 1998; Mallory and Vaucheret, 2010). The N-terminal PAZ domain has the sRNA-binding activity for the 3' end of the guide strand while the MID domain anchors the 5' monophosphorylated end. The PIWI domain harbors a catalytic site (Aspartate-Aspartate-Histidine, DDH) that functionally resembles an RNase H protein found in *Bacillus holodurans* (Song et al., 2004), and executes the endonucleolytic slicing of cognate RNA targets. The slicing activity has been demonstrated for the fission yeast Spo-AGO1, Drosophila Dme-AGO1 and Dme-AGO2, Arabidopsis Ath-AGO1, Ath-AGO4 and Ath-AGO7, and human Hsa-AGO2 (reviewed in Vaucheret, 2008). Interestingly, in humans, although Hsa-AGO3 has a DDH domain, it lacks slicing activity. This observation is suggestive that the presence of a DDH motif does not necessarily ensure slicing activity (reviewed in Vaucheret, 2008). In addition, Hsa-AGO2 has been reported to be involved in pre-miRNA processing, implicating its role in the miRNA biogenesis pathway (Diederichs and Haber, 2007; Cheloufi et al., 2010; Cifuentes et al., 2010).

There are 10 AGO proteins in Arabidopsis. Phylogenic analyses categorize them in three clades: AGO1, AGO5, and AGO10 within the first clade; AGO2, AGO3 and AGO7 within the second clade; and AGO4, AGO6, AGO8 and AGO9 in the third clade (Morel et al., 2002).

AGO1 is the founding member of the AGO family, and is the major effector protein in the miRNA-mediated gene silencing pathway (reviewed in Vaucheret, 2008; Voinnet, 2009).

AGO1 was initially identified by the pleiotropic developmental defects manifested by *ago1* mutants (Bohmert et al., 1998). Subsequent mutant screening studies implicated AGO1 in developmental programming and RNA silencing pathways (Fagard et al., 2000; Morel et al., 2002; Kidner and Martienssen, 2004; Yang et al., 2006b). Initial evidence suggesting that AGO1 plays a role in the miRNA pathway came from studies by Kidner and Martienssen, where they showed phenotypic similarity between an *ago1* mutant and *phb* (phabulosa) and *phv* (phavoluta) miRNA target gain-of-function mutants (Kidner and Martienssen, 2004). Since then, lines of evidence have demonstrated an essential role of AGO1 in miRNA function. First of all, in *ago1* mutants, miRNA accumulation is reduced, and reciprocally, levels of miRNA targets are increased (Vaucheret et al., 2004). Second, immunoprecipitation studies demonstrated that AGO1 bound to miRNAs (Baumberger and Baulcombe, 2005; Qi et al., 2005). Third, *ago1* mutants exhibit severe developmental phenotypes compared with other *ago* mutants, which display limited or no developmental defects (reviewed in Vaucheret, 2008). Last, but not least, AGO1 preferentially associates with small RNAs with a 5' uridine (Mi et al., 2008). Because most miRNAs have a 5' uridine, AGO1 is thought to be the major AGO protein in the miRNA pathway.

Regulation and turn-over of plant miRNAs

Biogenesis of plant miRNAs is subject to feedback regulation. For example, it has been shown that miR162 targets DCL1 transcripts for degradation (Xie et al., 2003), and AGO1 is regulated by miR168 (Vaucheret et al., 2006).

Over the past decade, many exogenous and endogenous mechanisms were characterized that suppress the small RNA functionality in post-transcriptional gene silencing (PTGS). Viral silencing suppressors were among the first to be identified (Roth et al., 2004; Qu and Morris, 2005). For example, the tombusvirus protein P19, the closterovirus protein P21, and the potyvirus protein HC-Pro inhibit silencing by sequestering anti-viral siRNAs (Lakatos et al., 2006). The cucumber mosaic virus protein 2b directly interacts with AGO1 to suppress the AGO1 slicing activity (Zhang et al., 2006d). The polerovirus protein P0 counteracts silencing by destabilizing AGO1 (Pazhouhandeh et al., 2006; Baumberger et al., 2007). Factors in the endogenous RNA degradation pathways such as the 5'-to- 3' exonucleases XRN4, XRN2 and XRN3, negatively regulate PTGS possibly via their 5'-3' exoribonuclease activities, to compete for access to the PTGS RNA substrates/templates (Gazzani et al., 2004; Souret et al., 2004; Gy et al., 2007).

In contrast to miRNA biogenesis, turnover of miRNAs has received relatively limited attention thus far. Due to their small size, it is generally believed that miRNAs are relatively stable molecules. Indeed, by triggering the inhibition of RNA POL II activity or depletion of miRNA processing enzymes, studies conducted in various animal systems have indicated that half lives of many miRNAs correspond to many hours or even days (van Rooij et al., 2007; Krol et al., 2010b). However, the slow turnover rate might not be a universal feature of miRNAs as in many cases miRNAs are required to bring about rapid changes in the transcriptome, thus requiring more active metabolism. Analogous to proteins, where the addition of a ubiquitin chain tags them for degradation, the stability of miRNAs can also be

governed by a similar mechanism. For instance, Yu et al. discovered that the addition of a methyl group to the 3’ end of miRNAs stabilizes the miRNAs while the 3’ uridylation of mature miRNAs may flag them for degradation (Li et al., 2005; Yu et al., 2005). Recently, a protein identified by Ramachandran and Chen has been suggested to act on the miRNA turnover pathway. It is a member of a 3' - 5' exoribonuclease protein family and thus was named small RNA degrading nuclease 1 (SDN1). SDN1 degrades small RNAs within a size range of 17 to 27 nt and yields products of 8-9 nt in length. This degradation is independent of the sequence of small RNAs. Thus, it is suggestive that SDN1 might act as the major enzyme regulating the steady-state level of miRNAs. AGO1, by contrast, may contribute to miRNA turnover simply by protecting miRNAs within the protein core, rendering them unavailable to exonucleases such as SDN1.

Regulatory roles of plant miRNAs

Plant miRNAs are involved in various aspects of plant life including developmental programming (reviewed in Zhang et al., 2006b; Voinnet, 2009), biotic and abiotic stress responses (reviewed in Sunkar, 2007; Ruiz-Ferrer and Voinnet, 2009), and secondary metabolite production (Gou et al., 2011; Ng et al., 2011; Robert-Seilaniantz et al., 2011). In Arabidopsis, for example, miR159 (Allen et al., 2007; Reyes and Chua, 2007; Alonso-Peral et al., 2012) and miR160 (Liu et al., 2007; Nonogaki, 2008) have been reported in several studies to be involved in hormonal signaling, regulating various biological programs such as germination and anther development. An antagonistic role of miR156 and miR172 has been reported to regulate plant juvenile-to-adult phase transition (Fornara and Coupland, 2009;

Poethig, 2009; Wu et al., 2009; Jung et al., 2011). In addition, miR172 has also been shown to be essential for floral identity establishment and flower development (Aukerman and Sakai, 2003; Chen, 2004). Recent advancement in Next-Gen sequencing technology has greatly facilitated the discoveries of roles of miRNAs in many stress conditions from a great array of species (reviewed in Sunkar, 2010). These deep sequencing studies have revealed potential roles of many miRNAs in abiotic stresses such as drought, salt, cold, excessive reactive oxygen species (ROS), and hypoxia. Moreover, many miRNAs also play a role in nutrient homeostasis. For instance, miR395(Jones-Rhoades and Bartel, 2004; Kawashima et al., 2009; Liang et al., 2010), miR398 (Yamasaki et al., 2007; Bouche, 2010; Zhu et al., 2011a), and miR399 (Aung et al., 2006; Bari et al., 2006; Lin et al., 2008; Hsieh et al., 2009a) have been shown to be involved in sulfate, copper and phosphate homeostasis, respectively. To generate a global view of the function of miRNAs in plants, I have summarized our current knowledge in Table 1.1.

It is worth noting here, that although many miRNAs are considered evolutionarily conserved, their expression and functions may vary in different organisms. A very well illustrated example is the case of miR156 in Arabidopsis and rice (Xie et al., 2012). In Arabidopsis, miR156 is dominantly expressed in the juvenile shoot and apex to target a group of SPL *(*SQUAMOSA-promoter Binding-like) transcription factors. SPLs regulate the expression of an array of important genes such as LEAFY, APETALA1, SOC1 (Suppressor of Overexpression of Constans1), TRICHOMELESS1, and interestingly, miR172 (Wu and Poethig, 2006; Wang et al., 2009b; Wu et al., 2009; Yamaguchi et al., 2009; Yu et al., 2010).

As Arabidopsis plants mature, the expression level of miR156 decreases gradually. Consequently, SPLs are de-repressed and start to activate genes that are essential in modulating the vegetative to reproductive phase transition. It has been known that the antagonizing roles of miR156 and miR172 are crucial for proper phase change in Arabidopsis (Wu et al., 2009). In rice, however, Xie et al. found that miR156 behaved in the opposite manner, as the level of miR156 increased in developing adult leaves (Xie et al., 2012). This observation was suggestive that miR156 in rice assumed a different role in regulating development, possibly as a facilitator, rather than a repressor, during the juvenile-to-adult phase transition.

Table 1.1 Summary of Arabidopsis miRNAs, the corresponding targets, and characterized functions

	miRNA	miRNA targets	Reference
Development events			
Leaf development, pattern and polarity establishment	miR165 miR166	HD-ZIP transcription factors: PHB,PHV, REV	(Juarez et al., 2004; Mallory et al., 2004b)
	miR164a	NAC transcription factors: CUC2	(Nikovics et al., 2006)
	miR319	TCP transcription factors: TCP2/3/4/10/24	(Palatnik et al., 2003)
	miR394	F-box protein: LCR	(Song et al., 2012)
	miR159	MYB transcription factors: MYB33/65	(Palatnik et al., 2003; Millar and Gubler, 2005)
Floral identity, flower timing and development	miR172	APETALA2-like transcription factors: AP2, TOE1/2/3	(Aukerman and Sakai, 2003; Chen, 2004; Schwab et al., 2005)
	miR164c	NAC transcription factors: CUC1/2	(Baker et al., 2005)
	miR159	MYB transcription factors: MYB33/65	(Achard et al., 2004; Millar and Gubler, 2005; Schwab et al., 2005)
	miR156	SBP-like transcription factors: SPL3	(Schwab et al., 2005)
	miR171	Scarecrow-like: SCL6-II, SCL6-III, SCL-IV	(Llave et al., 2002a)
Phase transition	miR172	APETALA2-like transcription factors: GL15	(Lauter et al., 2005)
	miR156	SBP-like transcription factors: SPL3/4/5	(Luo et al., 2006; Wu and Poethig, 2006)
Hormone signaling	miR159	MYB transcription factors: MYB33/65	(Achard et al., 2004; Schwab et al., 2005)
	miR160	Auxin responsive factors: ARF10/16/17	(Rhoades et al., 2002; Mallory et al., 2005; Wang et al., 2005; Liu et al., 2007)

Table 1.1 (Continued)

	miRNA	miRNA targets	Reference
Hormone signaling	miR167	Auxin Responsive Factors: ARF6/8	(Rhoades et al., 2002; Ru et al., 2006; Wu et al., 2006)
Stem cell identity	miR394	F-box protein: LCR	(Song et al., 2012)
Stomata development	miR824	MADS box protein: AGL16	(Kutter et al., 2007)
Enviromental stress repsonses			
Reactive Oxygen Species/Copper/zinc homeotasis:	miR398	Copper superoxide dismutases: CSD1/2	(Sunkar et al., 2006; Yamasaki et al., 2007; Beauclair et al., 2010)
		copper chaperone for superoxide dismutase: CCS1	(Beauclair et al., 2010)
Sulphate metabolism	miR395	ATP sulfurylases: APS1 /4	(Kawashima et al., 2009; Liang et al., 2010)
		Sulfate transporter: SULTR2;1	(Kawashima et al., 2009; Liang et al., 2010)
Phosphate homeostasis	miR399	E2 enzyme: PHO2	(Aung et al., 2006; Bari et al., 2006; Lin et al., 2008)
		Non-coding gene: IPS1	(Chitwood and Timmermans, 2007; Franco-Zorrilla et al., 2007)
Nitrogen starvation	miR393	bHLH transcription factors: AFB3	(Vidal et al., 2010)
other events			
Regulation of miRNA	miR162	miRNA biogenesis pathway: DCL1	(Xie et al., 2003)
	miR168	miRNA effector: AGO1	(Vaucheret et al., 2004)
Secondary metabolites	miR163	SABATH Methyltransferase: FAMT, PXMT1	(Ng et al., 2011)
	miR156	SBP-like transcription factors: SPL9/SPL15	(Gou et al., 2011)

Table 1.1 (Continued)

	miR393	bHLH transcription factors: AFB1	(Robert-Seilaniantz et al., 2011)
TasiRNA biogenesis	miR173	TAS1/2	(Allen et al., 2005; Montgomery et al., 2008b; Felippes and Weigel, 2009)
	miR390	TAS3	(Allen et al., 2005; Montgomery et al., 2008a; Marin et al., 2010)
	miR828	TAS4	(Chen et al., 2010)

Different modes of operation by animal and plant miRNAs

MiRNAs regulate protein synthesis post-transcriptionally by annealing to target mRNAs. In animals, most miRNAs form imperfect hybrids with target mRNAs within their 3'-untranslated regions (3'-UTRs). The "base-pairing" areas, usually termed the "seed regions", frequently are located between the 2nd to 8th nucleotide from 5'end of the miRNA. It has been known that it is the stringency of the base-pairing within the seed region that governs the regulatory specificity of miRNAs (reviewed in Eulalio et al., 2008; Filipowicz et al., 2008). By contrast, plant miRNAs exhibit near-perfect complementarity to their targets (reviewed in Bartel, 2004; Naqvi et al., 2012; Voinnet, 2009).

Stemming from the difference in miR:target sequence complementarity, it is generally accepted that (1) plant miRNAs usually target genes from the same family, while owing to the relaxed base-pairing requirements, animal miRNAs might have hundreds of targets with great sequence and functional diversity (Flynt and Lai, 2008) and (2) plant miRNAs

predominantly cause target cleavage, while animal miRNAs cause translational repression and mRNA deadenylation, followed by mRNA decay (reviewed in Vionnet, 2009; Cannell et al., 2008; Chekulaeva and Filipowicz, 2009; Wu and Belasco, 2008).

A preponderance of evidence from 5' RACE (5' rapid amplification of cDNA ends) and PARE (parallel analysis of RNA ends) studies is suggestive that miRNA-directed target cleavage occurs between nucleotides the 10 and 11 relative the 5' end of the miRNA (Dunoyer et al., 2004; German et al., 2008; Llave et al., 2002b; Souret et al., 2004). In plants, the extensive miRNA:target pairing encompasses nucleotides 9-11. Therefore the "slicing" mode of action is usually suggested for plant miRNAs (to see an example of central bulges preventing slicing, see Franco-Zorrilla et al., 2007). However, lines of evidence have also suggested the presence of translational regulation by plant miRNAs. Data from various experiments have shown that in some cases, although the transcript levels of miRNA targets remained unchanged, the corresponding protein expression diminished. Conversely, in mutant lines that were later identified to be defective in miRNA-directed translational repression, the level of both miRNAs and target transcripts were unaffected while the protein levels of many miRNA targets were elevated. Furthermore, central mismatches in miRNA:target hybrids that were thought to alter the slicing rate resulted in modest phenotypic consequences in several experiments (for example, Mallory et al. 2004b). Collectively, these data suggest that, in plants, miRNAs act to not only direct transcript cleavage, but also regulate the protein production from their mRNA targets (Aukerman and

Sakai, 2003; Chen, 2004; Gandikota et al., 2007; Brodersen et al., 2008; Dugas and Bartel, 2008; Beauclair et al., 2010; Yang et al., 2012).

Plant miRNAs act as translational repressors to modulate target protein synthesis

The first demonstrated case of miRNA-mediated translational repression in plants was reported by Aukerman and Sakai, 2003, and Chen, 2004, where miR172 predominantly acted as a translational repressor of APETALA2 (AP2) during Arabidopsis flower development. Since then, many studies have reported events where discrepancies between the extent of miRNA-directed transcript degradation and the loss of protein accumulation were observed. To date, miR398 (Dugas and Bartel, 2008; Beauclair et al., 2010), miR156/157 (Gandikota et al., 2007), and miR834 (Brodersen et al., 2008) are among the miRNAs that have been suggested to have a role in translational repression.

These observations were confirmed and broadened by the results obtained from a genetic screening and the subsequent characterization of miRNA-action deficient (*mad*) mutants in Arabidopsis (Brodersen et al., 2008). Characterization of these mutants suggested that most plant miRNAs acquired a regulatory mechanism involving both target cleavage and translational repression. Noticeably, neither the position (3'UTR, 5'UTR, or ORF) nor the degree of miR:target complementarity seemed to affect the prevalence of one mode over the other (Brodersen et al., 2008). These findings echoed the idea that transcript cleavage and translational repression entail similar sequence requirements (Palatnik et al., 2007). AGO1 is apparently involved in this translational control, as the hypomorphic *ago1-27* mutant exhibits

an elevated level of de-repressed target proteins (Brodersen et al., 2008). Interestingly, AGO10, the closest paralog of AGO1 in Arabidopsis, also seems to be a positive regulator of this process. A mutation in *AGO10* not only resulted in phenotypes partially resembling *ago1* mutants, but also suppressed the miRNA-imposed translational effects (Brodersen et al., 2008). However, the function of AGO1 and AGO10 in this regulatory pathway might not always be coupled because (1) the effects of compromised miRNA-imposed regulation in *ago1* and *ago10* mutants only partially overlap and (2) the expression domains and functions of AGO1 and AGO10 are not identical (Mallory et al., 2009; Zhu et al., 2011b). In Arabidopsis, other factors that have been shown to be required for miRNA-mediated translational repression include the microtubule-severing enzyme Katanin (KTN) and the P body component Varicose (VCS; Brodersen et al., 2008). Interestingly, in animals, similar requirements for tubulins (Parry et al., 2007) and decapping factors (Eulalio et al., 2007c) have been proposed for normal miRNA functions. Therefore, it seems to be an evolutionarily conserved mechanism for the cytoskeleton dynamics as well as the mRNA decay pathways to be involved in miRNA-directed translational repression.

Based on the existing data, Vionnet proposed a "two-layer" model where miRNAs bring their targets under translational as well as RNA stability control (reviewed in Vionnet, 2009). In this model, miRNA-imposed regulation can be divided into two separate states. In the first state, miRNAs predominantly regulate target expression via endonucleolytic cleavage, while in the second state, miRNAs operate to mainly repress the protein production of their targets. Regulation solely by cleavage or by translational inhibition is probably rare. Plant miRNA

activities, therefore, involve a combination of both of these two mechanisms. It is beneficial because, by combining different modes of actions, plant miRNAs offer a greater flexibility to modulate gene expression by either completely silencing target expression through directing mRNA degradation, or creating a reversible regulation of the corresponding protein syntheses. The latter mechanism is especially suitable in scenarios such as stressed growth conditions, where transient or reversible genetic regulation is required.

Models for miRNA-mediated translational repression

As aforementioned, unlike in plants, in animals, miRNAs act to regulate gene expression predominantly via their activities as translational repressors (reviewed in Bartel, 2004). To date, although some sporadic evidence may disagree, a consensus has been reached that the regulatory outcomes of animal miRNA function include translational repression, mRNA deadenylation and accelerated mRNA decay (reviewed in Nilsen, 2007). In fact, recent data suggest that these three mechanisms are not mutually exclusive, but rather sequential, with translational repression being the first mechanism, followed by mRNA deadenylation and ultimately degradation (Zdanowicz et al., 2009; Bazzini et al., 2012; Bethune et al., 2012; Djuranovic et al., 2012).

Regarding translational repression, although still largely open to debate, generally speaking, animal miRNAs contribute by the following hypothetical mechanisms (Figure 1.2): translational inhibition by blocking translation initiation (Figure1.2 B and Figure 1.2 C),

miRNA-mediated inhibition of translation elongation (Figure 1.3 D), and co-translational degradation of nascent polypeptide (Figure 1.3 E).

Although now it is well accepted that miRNA-meditated translational control is a widespread phenomenon in plants, mechanistically little is known about this process. It is partially due to the lack of knowledge and proper antibodies to detect the co-effectors that act in concert with miRISCs (miRNAs and the associated RISCs) in this regulatory pathway. Another obstacle that hinders our understanding stems from the fact that plant miRNAs exert their regulatory role, on a large scale, via causing target cleavage. Although it has been understood that globally, the miRNA-imposed translational regulation probably also plays a role toward plant miRNA activities, the translational effects brought about by this mode of miRNA action are usually masked by a greater effect sprung from the miRNA-mediated transcript cleavage. For the following sections, I have discussed possible models underlying this mechanism. Although most of the evidence used to support each model came from studies done in animal systems, because miRNA-mediated translational repression probably involves some evolutionarily conserved factors in plants and animals, knowledge obtained from animal studies might be beneficial in shedding light on understanding the scope of the mechanistic counterpart in plants.

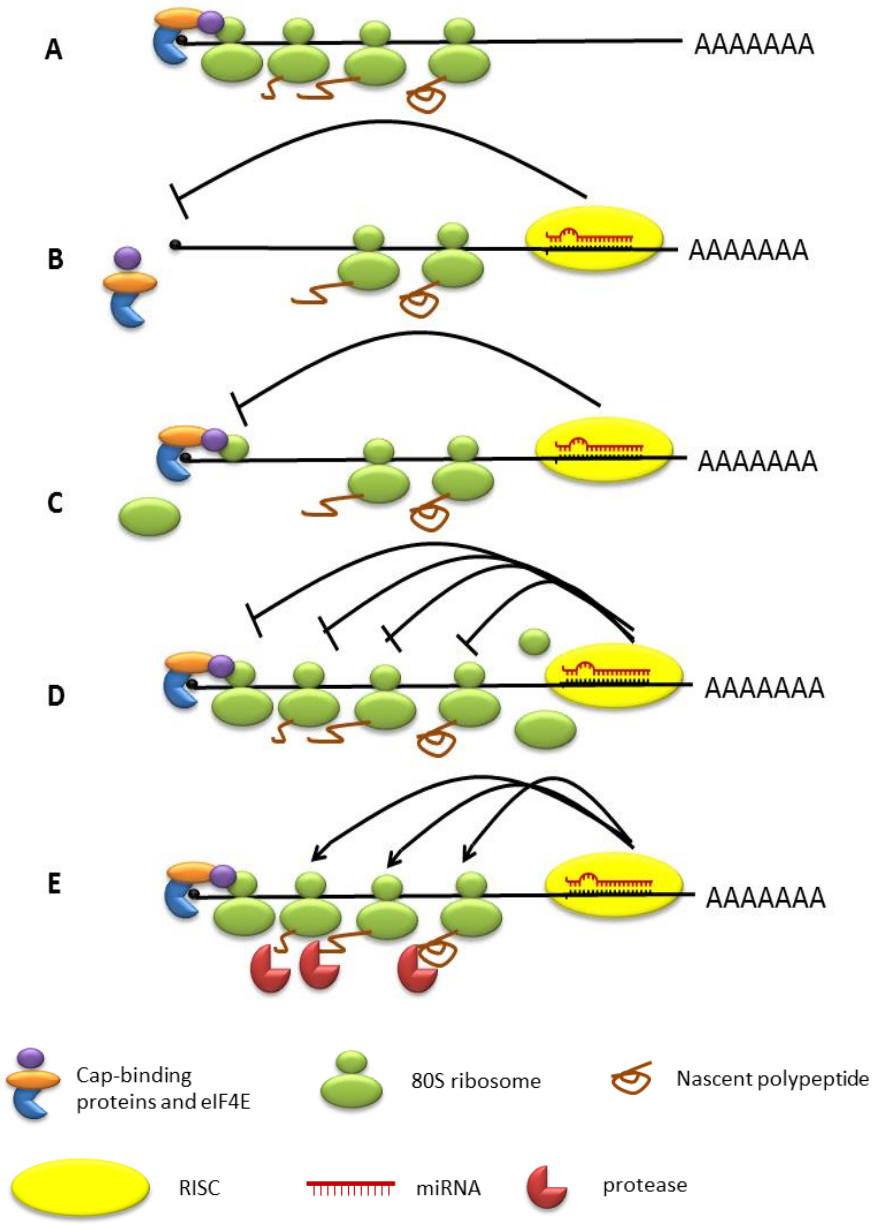

Figure 1.2 Hypothetical mechanisms of translational repression by miRNAs

A. mRNA undergoing normal translation; B. Inhibition of translation initiation by competition between RISC and eIF4E; C. Inhibition of translation at a step after cap recognition, such as blocking 40S and 60S subunit assembly; D. Inhibition of translation elongation, resulting in premature termination of peptide synthesis. E. Co-translational degradation of nascent polypeptides.

MiRNAs can inhibit translation at the initiation

Several major lines of evidence support a model where miRNAs inhibit translation at the initiation step. First of all, studies using sucrose density centrifugation to fractionate polysomes have revealed that the mRNAs repressed by miRNA activities were found to shift toward the top of the sedimentation gradient, indicating a reduced ribosome loading on these mRNAs (Huang et al., 2007; Selbach et al., 2008). Second, a very recent study has demonstrated that miRNA-mediated repression *in vitro* takes place during the 43S ribosome scanning (Ricci et al., 2012). Moreover, Ricci et al. have shown that poly-A binding proteins (PAPB) and the translation initiation factor eIF4G are essential for miRNA-mediated translation inhibition (Ricci et al., 2012). Third, Chendrimada et al. proposed that miRNAs interfere with target translation via RISC recruitment of eIF6 (Chendrimada et al., 2007). They showed that in both the Drosophila and *C. elegans* systems, depletion of eIF6 led to the abolishment of miRNA-mediated regulation of target protein production. Since eIF6 is a ribosomal protein known to be essential for translation initiation via its role in preventing the premature assembly of 80S ribosomes, they argued that the functional dependency of miRNAs on eIF6 activity was indeed an evolutionarily conserved mechanism acquired by miRNAs to interfere with the translational machinery (Chendrimada et al., 2007). Fourth, the observations that mRNAs containing internal ribosome entry sites (IRES) or nonfunctional caps are immune to miRNA-mediated translational repression suggest that miRNAs block an early step in translation, which presumably involves recognition of the m7GpppN cap (Humphreys et al., 2005; Pillai et al., 2005; Mathonnet et al., 2007; Thermann and Hentze, 2007).

MiRNAs can inhibit translation post-initiation

Biochemical evidence from animal studies have demonstrated that miRNAs and their targets can be associated with large polysomes (Lanet et al., 2009;Maroney et al., 2006; Nelson et al., 2004; Maroney et al., 2006; Nottrott et al., 2006; Olsen and Ambros, 1999; Petersen et al., 2006; Seggerson et al., 2002; Reynoso et al., 2012). Moreover, contradictory to observations made by Humpreys et al. and Pillai et al. (Humphreys et al., 2005; Pillai et al., 2005), Petersen et al. demonstrated that repression could occur in a 5'cap-independent manner for mRNAs engineered with an IRES (Petersen et al., 2006) . These findings suggest that miRNAs can block translation after initiation, and the inhibitive mechanism doesn't require the complete set of translation initiation factors, as the initiation at IRES requires only a minimum set of initiation factors (reviewed in Pisarev et al., 2005). In line with the post-initiation model, Nottrott et al. further argued that mammalian miRNAs might recruit proteases to translating polysomes to promote co-translational degradation of nascent polypeptides. They based their argument on the fact that, unlike in the controls, immunoprecipitation using antibody against the growing peptide failed to pull down the miRNA-repressed mRNAs (Nottrott et al., 2006). Interestingly, Selbach et al. found that genes categorized as "intrinsic to membrane" and "endoplasmic reticulum" (ER) were significantly overpresented among identified targets under the control of miRNA-mediated translational repression (Selbach et al., 2008). This finding raised a possibility that this miRNA-imposed translational control might occur preferably on membrane-bound polysomes, as a great portion of membrane-bound proteins are synthesized from polysomes that are anchored on the ERs.

In Arabidopsis, evidence has also shown the co-sedimentation of miRNAs and their targets with actively translating polysomes (Lanet et al., 2009). Moreover, this association is sensitive to treatments by the elongation inhibitor puromycin and micrococcal nucleases, which is suggestive that this miRNA association with polysomes truly reflects the functional interaction between miRNA activities and the translational machinery. Furthermore, hypomorphic *ago1-25* mutants with impaired AGO1 function exhibit loss of association of miRNAs with polysomes, which is indicative that the miRISC functionally interacts with polysomes to regulate target translation (Lanet et al., 2009). Interestingly, Ma et al. reported that in Chlamydomonas, some siRNAs could function as miRNAs to inhibit protein synthesis without apparent mRNA destabilization. These siRNAs are also found to be associated with polysomes. Intriguingly, they appear to have a reduced sensitivity to treatment by translation inhibitors such as cycloheximde. These data suggest that, at least in Chlamydomonas, siRNA-mediated translational control might involve changes to the function or/and conformation of translating ribosomes (Ma et al., 2013).

Processing bodies are involved in miRNA-mediated regulation

Processing bodies (P bodies) have been shown to be the cytoplasmic foci where multiple biological processes including mRNA decapping, deadenylation, and mRNA 3'-5' decay take place (reviewed in Franks and Lykke-Andersen, 2008; Parker and Sheth, 2007). P bodies are dynamic, and their assembly is dependent on, and usually proportional to, the pool of nontranslating mRNAs (Liu et al., 2005a; Teixeira et al., 2005). In animal systems, a preponderance of evidence has suggested the important role of P bodies in miRNA function.

First of all, miRISCs and translationally repressed mRNAs have been shown to be co-localized to P bodies (Liu et al., 2005a; Chan and Slack, 2006; Eulalio et al., 2007a). Second, many components in the miRNA silencing machinery have been reported to be present in P bodies (Liu et al., 2005a; Bhattacharyya et al., 2006; Leung et al., 2006; Leung and Sharp, 2007; Pare et al., 2009). It has been proposed that P bodies are the loci where miRNA-repressed mRNAs can be stored and further processed (Liu et al., 2005b; Leung et al., 2006; Pare et al., 2009). Third, in mammals, the P body component GW182 has been shown to be essential for miRNA-directed target repression. Depletion of GW182 abrogates miRNA-mediated regulation (Liu et al., 2005b; Chan and Slack, 2006; Eulalio et al., 2007a). However, it is not likely that the P body is the factor that triggers the miRNA-mediated silencing mechanism. Eulalio et al. demonstrated that (1) P body formation was not required for miRNA-mediated silencing; (2) blocking the miRNA biogenesis pathway prevented P body formation; (3) releasing mRNAs from polysomes was not sufficient to trigger P body assembly: polysome-free mRNAs must enter either the silencing or decapping pathway to nucleate P bodies. Therefore, it is very likely that the aggregation of nontranslating mRNAs into P bodies is the consequence of, not the reason, for many types of translational control (Eulalio et al., 2007b).

Interestingly, in plants, the P body component VCS has been identified as a factor that is involved in miRNA-directed translational repression (Brodersen et al., 2008). In addition, VCS and two other Arabidopsis P body components, namely DCP1 and DCP2 (decapping 1 and 2), have been shown to be involved in miRNA biogenesis, as *vcs, dcp1* and *dcp2* mutants

exhibit reduced miRNA accumulation (Motomura et al., 2012). All of these findings suggest that P bodies indeed are robustly involved in miRNA-mediated regulatory pathways.

Several lines of evidence have suggested a highly dynamic interchange of mRNAs between polysomes and P bodies. First, inhibition of translation initiation by various drug or stress conditions causes loss of mRNAs from polysomes and a corresponding increase of mRNAs in P bodies (Kedersha et al., 2005; Teixeira et al., 2005; Anderson and Kedersha, 2006). Second, freezing mRNAs in polysomes by blocking translation elongation reduces the formation of P bodies. These findings suggest that mRNAs in these two compartments are maintained in a dynamic equilibrium (Cougot et al., 2004; Teixeira et al., 2005). Third, mRNAs that are arrested in P bodies have been shown to return to translation in both yeast and mammalian cells (Brengues et al., 2005; Bhattacharyya et al., 2006).

Interestingly, quantitative analysis of AGO2 protein distribution in mammalian cells revealed that AGO2 localizes to both the diffuse cytoplasm and P bodies (Leung et al., 2006). Intriguingly, after blocking of translation initiation by an initiation inhibitor, a significantly increased accumulation of AGO2 at P bodies was observed (Leung et al., 2006). Therefore, consistent with the dynamic interchange of mRNAs between polysomes and P bodies, the miRNA silencing machinery is also robustly involved in shuttling between these two cellular compartments. These findings further support the notion that P bodies and polysomes are the cytoplasmic loci for miRNA activities: miRNAs firstly act to repress actively translating

polysomes, and then the miRISC-target ribonucleoprotein (RNP) complexes are shuttled to P bodies for further processing.

Reconciliation of discrepancies in experimental data and models for miRNA-mediated translational control

Based on a comprehensive analysis of existing literature, Morozova et al. generated a mathematical model where they assumed all of the miRNA modes of action coexisted (Morozova et al., 2012). In this model, the regulatory mechanism detected in any given experiment was thought to be an outcome of all the intrinsic characteristics (parameters) of the target mRNA and its associated biological processes. Computational interrogation of this model suggested that for a given mode of miRNA action to be detected, it needed to be targeting the rate-limiting step of the translation mechanism in the biological context (Morozova et al., 2012). In other words, what seemed to be an outcome of one single regulatory mechanism might actually be the result of multiple concurrent mechanisms, with the rate-limiting mechanism being dominant. This model properly put many of the existing experimental data in line: although they initially seemed to be contradicting one another, when factoring in all the parameters from various experimental designs, the majority of those data became suggestive that miRNA regulatory mechanisms co-exist. For example, evidence that supports the "initiation model" usually was collected from artificial systems such as cell extracts (Thermann and Hentze, 2007; Ricci et al., 2012) and cell lines transfected with engineered reporter genes (Humphreys et al., 2005; Pillai et al., 2005). In contrast, with few exceptions (Nottrott et al., 2006; Petersen et al., 2006), biochemical evidence that supports

the “post-initiation model” frequently comes from studies of endogenous miRNAs and targets, from both animal (Kim et al., 2004; Nelson et al., 2004; Maroney et al., 2006) and plant systems (Lanet et al., 2009; Reynoso et al., 2012).

Plant miRNA-mediated translational regulation

In Figure 1.3, I summarized our current knowledge of miRNA-mediated translational regulation in plants. The decapping factors VCS, DCP2 and DCP2 have been characterized as P body components in plants (Xu et al., 2006). Although the P body has not been directly demonstrated in plants to be essential for miRNA-mediated translational control, lines of evidence strongly support this notion. First of all, the plant P body component VCS has been implicated in this regulatory pathway as *vcs* mutants displayed de-repression of miRNA-regulated inhibition of protein production (Brodersen et al., 2008). Moreover, VCS and the other two characterized P body components, DCP1 and DCP2, have been indicated in the miRNA functional pathway (Motomura et al., 2012). Second, the GW-rich protein SUO is a potential analog of the human GW182 protein and has been proven to be essential for miRNA-mediated translational repression in plants. It has been shown to be localized in P bodies (Yang et al., 2012).

Even though the biochemical evidence has shown that plant miRNAs co-localize with polysomes and this association is very likely a result of the functional interaction between miRNAs and their targets during translation (Lanet et al., 2009), no evidence so far has been produced to decipher mechanistically how plant miRNAs contribute to target regulation at

the level of translation. Therefore, in my project, I aimed to study (1) which miRNA species are potentially involved in this regulation; (2) possible mechanisms that miRNAs employ in this regulatory pathway.

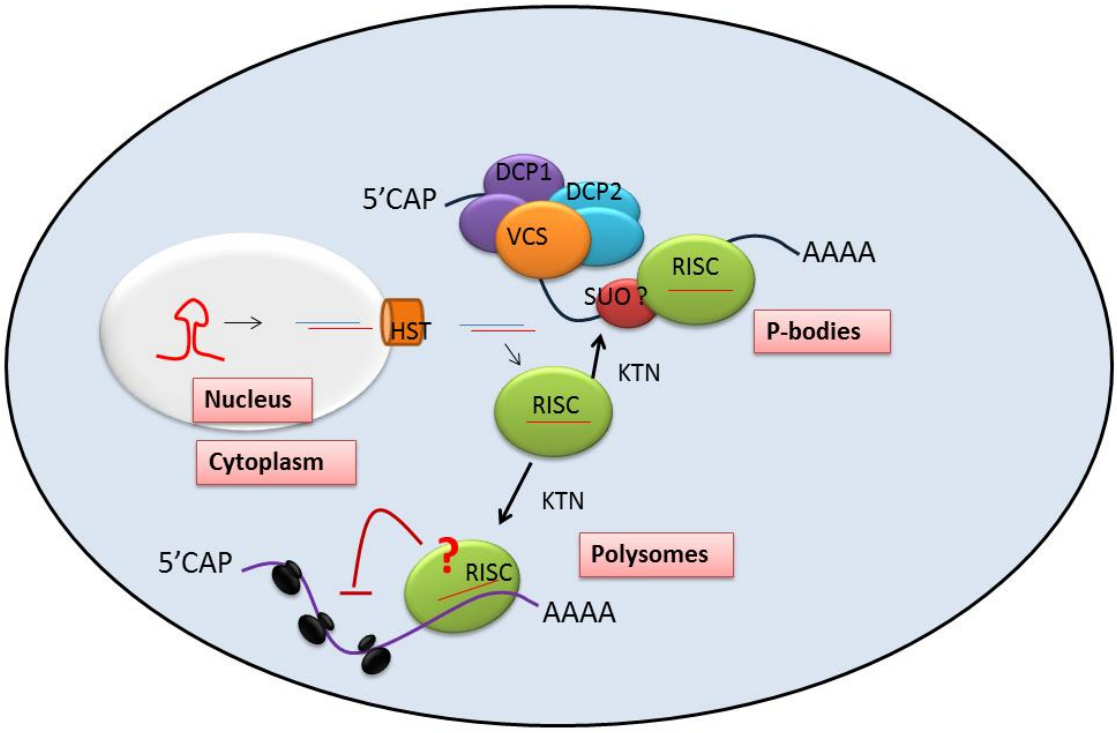

Figure 1.3 Illustration of known factors involved in plant miRNA-mediated translational repression

There are two major types of miRNA-directed post-transcriptional regulation. (1) target cleavage and (2) translational repression. Only the scenario of miRNA-mediated translational control is shown here. Once miRNA is incorporated into the miRISC, it can be shuttled to some cytoplasmic compartment such as P bodies, where it presumably directs mRNA target degradation. It can also bind to polysomes to regulate target translation. These two mechanisms probably work in concert to bring about the desired gene regulation.

Thesis Outline

In Chapter 2, I described the deep sequencing study I performed to identify miRNAs that were preferentially associated with polysomes in maize endosperm. There, I presented data that suggested that some miRNAs might bear an intrinsic preference to associate with polysomes while other miRNAs predominantly remained in ribosome-free fractions. Furthermore, my data suggested the presence of a potentially novel trans-acting siRNA regulatory pathway, and that some previously uncharacterized miRNA* s might contribute to gene regulation specific to maize endosperm.

In Chapter 3, I carried out miRNA RT-qPCR analyses and verified the results I obtained from the Next-Gen study in Chapter 2. Moreover, I profiled miRNA expressions at different developmental stages from both maize endosperm and embryos. Data obtained from these studies suggested that miRNAs were robustly involved in regulating gene expression to meet various developmental and physiological needs and some of this miRNA-imposed regulation may occur co-translationally. To gain a mechanistic insight into the miRNA-mediated translational control in plants, I performed several immunoprecipitation studies using a transgenic Arabidopsis line overexpressing a tagged ribosomal protein. By doing that I proved that AGO1was associated with polysomes *in vivo*, and the extent of this interaction varied at different developmental stages. In this chapter, based on our data and previous findings, I proposed a model where plant miRNAs contribute to gene regulation by interacting with the translational machinery.

In the supplemental data, I included information such as figures and sequence alignments that were important but not included in Chapters 2 and 3.

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Chapter 2

Small RNA Next-Gen Sequencing Reveals a Distinctive Small RNA Population in Polyribosomes from Immature Maize Endosperm

Abstract

Deep sequencing studies from various organisms have implicated the robust roles of miRNAs during seed development. In this study, we were interested in investigating the putative roles of various miRNAs during maize endosperm development. Particularly, we sought to understand the functional interactions between miRNA activities and the translational machinery in plants. Based on previous research, we hypothesized that miRNAs could act as translational repressors to negatively regulate the protein production from cognate target transcripts. To test our hypothesis, we performed a Next-Gen sequencing study to identify all of the miRNAs in maize endosperm harvested 18 DAP (Days After Pollination). Specifically, by employing sucrose density differential ultracentrifugation, we isolated miRNAs that were associated with ribosomes from the ribosome-free fractions. Our Next-Gen sequencing data suggested that some microRNA populations were preferentially associated with ribosomes. Moreover, our study also revealed some interesting phenomena that seemed to be present only in maize endosperm. These observations involved the presence of previously uncharacterized miRNA*s and tasiRNA-triggering miRNAs.

Introduction

Maize endosperm and genomic imprinting

Maize endosperm is one of the most significant agronomic tissues because it is not only a source of food and feed, a feedstock for industrial manufacturing of sweeteners, beer and soft drinks, but also a raw material for biofuel/ethanol production. From a biological perspective, endosperm is an interesting study model because it is triploid, inheriting one paternal and two maternal sets of chromosomes.

Development of maize endosperm has several distinct phases: (1) early development, including double fertilization, syncytium formation and cellularization; (2) differentiation, wherein important events such as the formation of the four main cell types (transfer cells, aleurone, starchy endosperm, and embryo-surrounding cells), the periods of mitosis and endoreduplication and the accumulation of storage compounds occur; and (3) maturation, featured by programmed cell death (PCD), seed dormancy and desiccation (reviewed in Sabelli and Larkins, 2009). .

Early endosperm development starts with the formation of the triploid primary endosperm nucleus resulting from double fertilization. Within four days after pollination (DAP) syncytium and endosperm cellularization are already visible. Then the endosperm undergoes a series of mitotic cell proliferation and endoreduplication events, which coincide with the accumulation of the starch and protein reserves. By 16 DAP, the average DNA content and nuclei number enter a plateau stage while the fresh weight keeps increasing (reviewed in

Sabelli and Larkins, 2009). Meanwhile, PCD in maize starchy endosperm starts to take place around 16 DAP. By 28 DAP and well before seed maturation is completed, the upper half of the endosperm is dead (Young and Gallie, 2000; Sabelli and Larkins, 2009).

Because of the 2:1 ratio of maternal: paternal genome dosages in endosperm cells, development of the endosperm is thought to be largely under maternal genetic control (Kowles et al., 1997; Wang and Larkins, 2001). However, recent studies have suggested that proper endosperm development is also a result of complex genetic and epigenetic interactions. One example is genomic imprinting, a largely endosperm-specific phenomenon by which a subset of genes is expressed in a parent-of-origin-dependent manner (Bauer and Fischer, 2011; Luo et al., 2011). In maize, as in Arabidopsis (Gehring et al., 2011; Hsieh et al., 2011; McKeown et al., 2011) and rice (Luo et al., 2011), and other previous reports (Raissig et al., 2011), an analysis of large-scale sequencing data of 10 DAP endosperm transcriptomes revealed the presence of widespread uniparental imprinting events on both non-coding and protein-coding RNAs (Zhang et al., 2011a).

Parent-of-origin-specific expression of genetically equivalent alleles is achieved by specific epigenetic modifications. Single gene studies as well as genome-wide imprinting analyses have provided knowledge regarding mechanisms of genomic imprinting in plants. Genomic imprinting is a consequence of changes in DNA methylation in endosperm (Gutierrez-Marcos et al., 2006; Jullien et al., 2006; Kohler et al., 2012). In plants, DNA methylation occurs in three sequence contexts: symmetric CG and CHG methylation as well as the

asymmetric CHH methylation (where H= C, G, or A; Kohler et al., 2012). While the conserved methyltransferase MET1 and the plant-specific methyltransferase CMT3 are responsible for maintaining CG and CHG methylation, respectively (Feng et al., 2010; Law and Jacobsen, 2010; Zemach et al., 2010), the asymmetric CHH methylation is established and maintained by the RNA-dependent RNA polymerase pathway, in which the DNA methyltransferase DRM2 acts in association with AGO4 and 24-nt siRNAs to direct DNA methylation on targeted chromatic regions (Law and Jacobsen, 2010).

Interestingly, Mosher et al. and Lu et al. demonstrated a predominantly maternal origin of siRNAs in Arabidopsis endosperm (Mosher et al., 2009; Lu et al., 2012). This group of siRNAs is generated by the plant-specific RNA POL IV, which preferentially transcribes RNAs from transposons or heterochromatic regions (Gendrel and Colot, 2005; Ramachandran and Chen, 2008a). The RNA POL IV derived transcripts are converted to double-stranded RNA by RNA-dependent RNA POL II, further processed into 24-nt siRNAs by DCL3, then bound by AGO4 to guide the *de novo* methylation of cytosines in CHH sequence contexts by the DNA methyltransferase DRM2 (Law and Jacobsen, 2010). Since global hypomethylation was observed during endosperm development (Jullien et al., 2008; Gehring et al., 2009; Hsieh et al., 2009b), it was proposed that reactivated transposons gave rise to the 24-nt siRNA group, which not only generated a wave of *de novo* DNA methylation in endosperm, but migrated to the embryo, reinforcing the proper gene methylation and silencing in the germ line (Jullien et al., 2008; Hsieh et al., 2009b).

MiRNAs and current miRNA studies in maize seeds

In addition to the aforementioned 24-nt siRNAs, there is another major group of small RNAs that have been extensively studied, namely the miRNAs. MiRNAs are regulators that play essential roles in many diverse biological processes such as development, metabolism and apoptosis (Bartel, 2004). They are generally transcribed by RNA POL II to primary miRNAs (pri-miRNAs; Bartel, 2004).

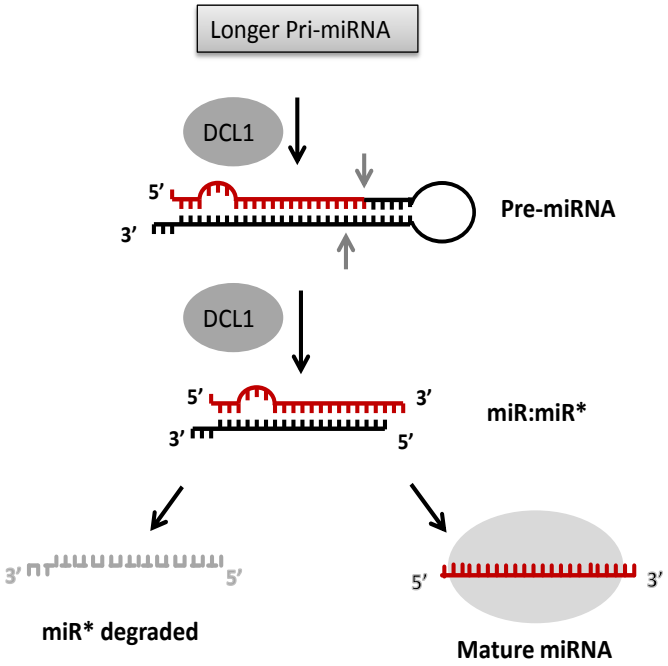


Figure 2.1 Structural illustrations of pre-miRNA and miRNA:miRNA* duplex.

As shown in Figure 2.1, following transcription, pri-miRNAs are processed in a sequential manner by DCL1 into firstly the stem-loop precursors (pre-miRNAs) and then the miRNA:miRNA* duplexes (Sun; Reinhart et al., 2002; Xie et al., 2005; Zhang et al., 2006b). The miRNA:miRNA* duplexes in plants are methylated on the 3' termini by HEN1(Yu et al., 2005) and transported to the cytoplasm by the Exportin-5 homolog HASTY (Bollman et al., 2003; Park et al., 2005). Mature miRNAs are loaded into RNA-induced silencing complexes (miRISCs) to guide the cleavage or translational repression of target mRNAs based on sequence complementarity, while the miRNA*s are gradually degraded (Brodersen et al., 2008; Brodersen and Voinnet, 2009; Sun, 2011; Zhang et al., 2006b;). Originally reported in 2002 in Arabidopsis, miRNAs are usually 20-22 nt in length (Llave et al., 2002a). Unlike their animal counterparts, plant miRNAs exhibit near-perfect sequence complementarity to their targets (Bartel, 2004; Jones-Rhoades et al., 2006). Two major miRNA regulation mechanisms have been suggested in plants: mRNA cleavage and translational inhibition (Aukerman and Sakai, 2003;Gandikota et al., 2007; Brodersen et al., 2008). To date, most of the identified targets of plant miRNAs are transcripts encoding transcription factors that are involved in developmental patterning, cell differentiation and stress responses (Rhoades et al., 2002; Phillips et al., 2007; Sunkar et al., 2007).

As shown in Figure 2.2, one unique miRNA population that later becomes particularly interesting in our study is the trans-acting siRNA (tasiRNA). TasiRNAs are genetically defined at specific loci and arise in a phased manner, by the DCL4 processing of the dsRNA generated from RNR6/SGS3 activity on RNA POL II transcripts (Allen et al., 2005). To

trigger tasiRNA production, an initial target cleavage by miRNAs is needed. Once generated, tasiRNAs are loaded to miRISCs and guide the target cleavage via the same pathway as do regular plant miRNAs (Vazquez et al., 2004b; Allen et al., 2005). It has been reported that it is the miRNAs with the length of 22 nucleotides, rather than the 21-nt miRNAs, that are responsible for re-programing the miRISCs into tasiRNA-generating pathways (Cuperus et al., 2010). Moreover, Manavella et al. provided evidence demonstrating that the tasiRNA transitivity can actually be triggered when either strand of the miRNA/miRNA* duplex is 22-nt (Manavella et al., 2012).

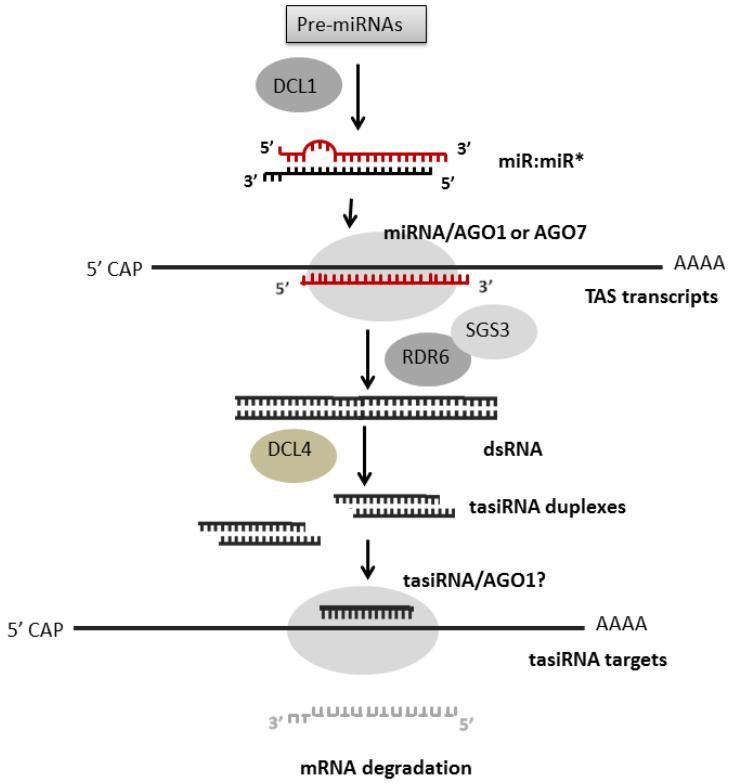


Figure 2.2 The biogenesis pathway of trans-acting siRNAs

Several deep sequencing studies of miRNAs from maize seeds have been reported over the past couple of years. In 2011, Wang et al. performed Next-Gen sequencing of a small RNA library constructed from maize embryos 24 hr after imbibition and identified 115 known miRNAs (zma-miRNAs) and 167 novel miRNAs (Wang et al., 2011a). Later, by comparing miRNA expression in dry embryos and imbibed embryos, the same group identified miRNA families that were significantly up-regulated or down-regulated after 24hr of imbibition. Based on these observations and the computational prediction of miRNA targets in maize,

they concluded that carbohydrate catabolism genes were specifically enriched in dry seeds, possibly in the form of storage mRNAs during seed desiccation, while in imbibed seeds, the elevated miRNA targets covered a broad spectrum of functional categories including genes involved in amino acid biosynthesis, isomerase activity, and ligase activity (Li et al., 2013). MiRNA profiling during maize seed development has also been reported. In a previous study, total RNA from immature maize seeds at the stages of 10, 15, 20, 25, and 30 DAP was pooled and submitted for deep sequencing. Along with 54 novel maize miRNAs, 125 known miRNAs were identified (Kang et al., 2012). MiRNA expression data collected from these studies will be discussed in parallel to my deep sequencing results later in this chapter.

Translational repression by miRNAs in plants

The near-perfect sequence complementarity between plant miRNAs and their targets renders the permeating belief that, unlike in animal systems, in plants, miRNAs predominantly regulate targets by endonucleolytic cleavage via the "slicer" activity of ARGONAUTE proteins. However, the fact that the target cleavage is more readily observed in plants doesn't nullify a role for miRNA-directed translational repression. In fact, events where specific plant miRNAs act to maintain targets in a translationally inert state have been reported since the early years of miRNA functional characterization (Aukerman and Sakai, 2003; Chen, 2004; Gandikota et al., 2007; Dugas and Bartel, 2008; Beauclair et al., 2010). More recently, novel endogenous protein factors, such as the glycine-tryptophan-rich protein (GW protein) SUO, have been shown to be essential for miRNA-directed translational repression (Yang et al., 2012). Interestingly, a large-scale mutant screening for *mad* (*miRNA action defective*)

mutants revealed the necessity of the microtubule severing protein KTN, the decapping factor VCS and the AGO1 homolog AGO10 in sustaining proper inhibition of target protein synthesis (Brodersen et al., 2008). These findings led the authors to suggest that the mRNA degradation pathways, as well as microtubule dynamics, were involved in this regulatory mechanism (Brodersen et al., 2008). Using differential centrifugation through sucrose gradients, Lanet et al. demonstrated the co-sedimentation of miRNAs and their associated protein complexes (miRISCs) with polyribosomes (Lanet et al., 2009). In addition, puromycin, a chemical known to dissociate active polysomes, not only dissociated polysomes but also released the miRISCs from them. These findings are suggestive that miRNAs potentially execute their functions as translational repressors by directly interfering with the translation machinery (Lanet et al., 2009).

As summarized above, in plant systems, events of miRNA-directed translational repression and factors that are involved in this mechanism have just started to emerge over the past few years. Unlike in animal systems, where miRNAs predominantly act to repress target translation, in plants, it is still under debate how significant the miRNA-directed translational repression is for miRNA activities. Compared to null mutants of DCL1 (protein that acts on the miRNA biogenesis pathway, see Chapter1 Figure 1.1), which are lethal very early in embryogenesis (Schauer et al., 2002), the *suo* mutants that are potentially defective in miRNA-mediated translational repression display only minor developmental defects (Yang et al., 2012). Yang et al. argued that the subtle phenotype might suggest a less important role of translational inhibition for miRNA activities than target cleavage in plants. However, they

could not exclude the possibility that the GW-repeat protein SUO was responsible for the activity of only some miRNAs. An exhaustive study to identify GW-repeat proteins in Arabidopsis revealed the presence of 20 candidate genes that are evolutionary conserved between animals and plants (Karlowski et al., 2010). Therefore, the relatively weak phenotype of *suo* mutants might, in fact, be attributed to the functional redundancy of the GW-repeat proteins in plants.

In animals, miRNAs have been suggested to repress gene expression via several mechanisms: (a) inhibition of translation initiation; (b) inhibition of translation elongation; and (c) mRNA degradation (Nilsen, 2007; Pillai et al., 2007; Eulalio et al., 2008b; Huntzinger and Izaurralde, 2011). Although the presence of each of the respective mechanisms was revealed through different lines of evidence, it is very likely that at least in some cases, they act in concert to regulate gene expression. In fact, it was shown that mRNA degradation by miRNAs was the outcome of the miRNA-directed inhibition of translation (Djuranovic et al., 2012). In plants, several lines of evidence support the functional interactions between many miRISCs and the actively-translating polysomes (Lanet et al., 2009). These findings indicated the presence of a post-initiation mechanism by which plant miRNAs regulate the cognate gene expression, possibly resembling what has been described in the animal system.

There are many angles from which one can address the question of miRNA-mediated translational repression. One of the most straightforward approaches would be to test for the

co-presence of miRNAs and their targets in the translation machinery. Around 18 DAP, the cell proliferation by mitosis has diminished with programmed cell death just starting to take place. While the genomic DNA contents reach a peak at 18 DAP, the kernel fresh weight continues to increase drastically due to the active synthesis of starch and storage protein. These unique traits render the 18 DAP maize endosperm a proper system to study the miRNA-mediated translational repression. In the present work, based on our current knowledge, we hypothesized that plant miRNAs act as translational repressors and exert their repressive roles by directly interfering with translation machinery. To test this hypothesis, by using 18 DAP maize endosperm, we sought to identify miRNAs that were preferentially associated with ribosomes by Next-Gen sequencing. Our data revealed that, although to various degrees, many miRNA species indeed were found to be associated with ribosomes. Interestingly, our data also suggested the presence of a potentially novel trans-acting siRNA regulatory pathway, and that some previously uncharacterized miRNA* s might contribute to gene regulation specific to maize endosperm.

Results

Small RNA sequencing

If miRNA is involved in translational repression, it should be associated with the translational machinery, the ribosomes. To identify miRNAs associated with ribosomes, we isolated small RNA (sRNA) from the ribosomal fraction of immature maize endosperm harvested at 18DAP. This sRNA was used to construct an sRNA library and sequenced with the Roche 454 Next-Gen sequencing platform. Figure 2.3 represents the methodology used for sample preparation and sRNA library construction. In brief, ribosome-associated RNAs (will be referred to as the ribosomal fraction) and ribosome-free RNAs (will be referred to as the non-ribosomal fraction) were separated by differential centrifugation through a sucrose pad. sRNAs were isolated from these two fractions and used to construct the cDNA library of sRNAs. Four libraries from two biological replicates were barcoded and submitted for 454 sequencing. A total of 483,312 reads remained after removal of low quality sequences. Mapping of sRNAs within the range of 18nt to 30nt to the maize transcriptome database (B73 RefGen_v2, release 5b.60) indicated that a total number of 111,292 reads matched perfectly to the maize genome, accounting for 64% of the total sRNA counts in that length range. Moreover, a total number of 62,901 reads had one nucleotide that differed in their sequences from the maize genome.

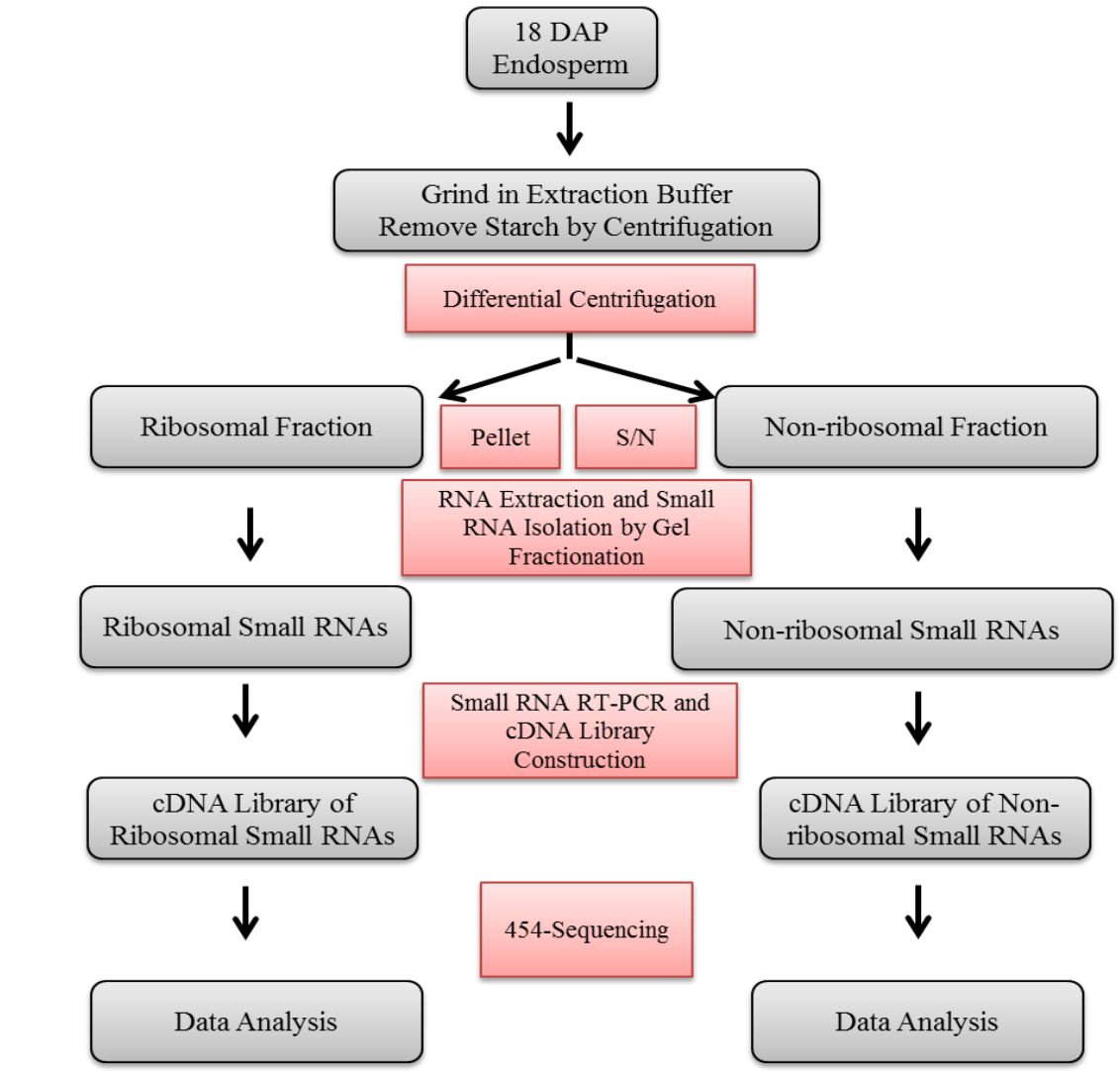


Figure 2.3 Schematic of sample preparation and sRNA library construction

After the removal of starch by centrifugation, the ground endosperm was subjected to differential centrifugation to separate ribosome-associated RNAs from ribosome-free RNAs. RNAs were extracted by TRIzol LS (Invitrogen, CA) and small RNAs were isolated by polyacrylamide gel fractionation. Small RNAs then were ligated to adaptors, barcoded and amplified via RT-PCR to generate cDNA libraries for each sample, respectively. Libraries were pooled and submitted for 454-sequencing.

The sRNA size distribution in the ribosomal and non-ribosomal fractions displayed distinctively different patterns (Figure 2.4 A). In the non-ribosomal fraction, most sequenced sRNAs were 24-nt in length. In the ribosomal fraction, the abundance of sRNA species less than 20 nt drastically increased compared to that in the non-ribosomal fraction, with the most changed species being the 16-nt sRNA population. To compare the size distributions in the two different biological replicates, sRNAs ranging from 18 to 30 nt in length from the 4 libraries were further analyzed. The length distributions of these sRNA species from two biological replicates are shown in Figure 2.4 B and Figure 2.4 C. Even though there are some differences possibly due to the natural variation in the two different biological replicates, a general pattern persists. Among the non-ribosomal sRNA populations, the most abundant sRNA species was 24-nt in length, accounting for almost 30% of the total small RNA population (18-nt to 30-nt). It is followed by sRNAs with the lengths of 21 nt (16%) and 22 nt (12%), which were the lengths of canonical miRNAs. In ribosomal fractions, however, the prominent 24-nt peak no longer was present. For the 21-nt to 24-nt ribosomal sRNA populations, when compared to the corresponding populations in the non-ribosomal fraction, the peak shifted from 24 nt to 21 nt. This change of length distribution in sRNA populations is consistent with an expected differential distribution of miRNAs and siRNAs in these two sample fractions: miRNAs usually take the signature forms of 21 and 22 nt while the 24-nt species is likely to be comprised of the heterochromatic endogenous siRNAs.

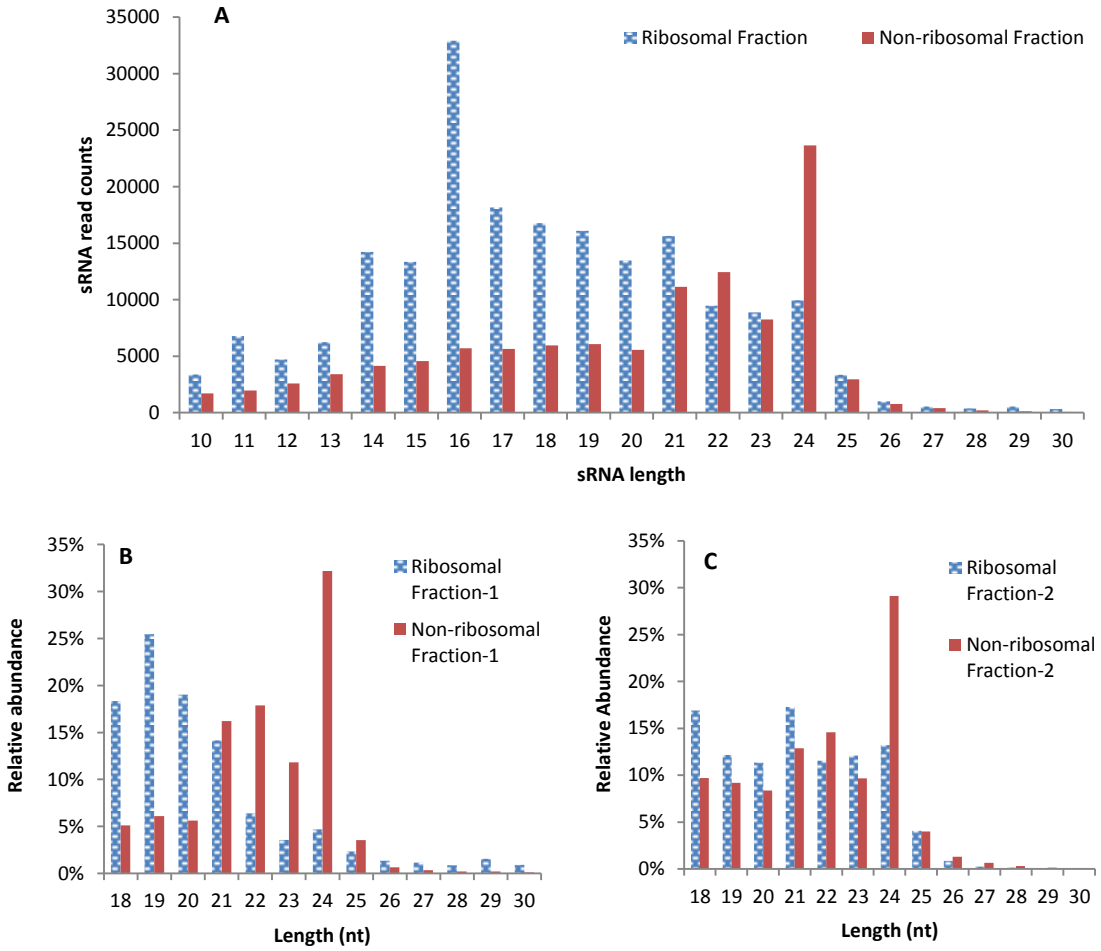


Figure 2.4 Length distribution and sequence abundance of ribosomal and non-ribosomal sRNAs in 18DAP maize endosperm.

A: Total sRNA abundance in each sRNA size category; Histogram presentation of sequence-length distribution for reads in ribosomal and non-ribosomal RNA libraries. The x-axis indicates sequence sizes from 10 nt to 30 nt. The y-axis indicates the total number of reads for each given size group. B: Relative abundance of each sRNA size category in biological replicate 1; C: Relative abundance of each sRNA size category in biological replicate 2. The Y axis indicates the percentage of ribosomal or non-ribosomal reads for every given length (Panels B and C).The histogram was generated by dividing the read counts of each given length by the total read counts (18-nt to 30-nt) in each individual library.

*Note that for each sRNA species, the levels in ribosomal and non-ribosomal fractions are not directly comparable due to the lack of a common normalizer between these two fractions. Thus, one can compare only across the abundance of different sRNA species within the same library.

To generate data on all the functional RNA groups present in this study, sRNAs within the range of 16-nt to 30-nt were mapped to corresponding RNA databases with a tolerance of one mismatch. Four individual libraries were analyzed. Reads of sRNAs that matched rRNAs (LSU rRNA, as shown in Figure 2.5 A and table 2.1), tRNAs (Figure 2.5 B and Table 2.1) and maize transcriptomes (Figure 2.5 C) were categorized by their sizes. The relative abundance of each analyzed functional RNA group is shown in Figure 2.4 and Table 2.1. Unexpectedly, an over-representation of rRNAs was observed in the ribosomal fraction from biological replicate 1. The total sRNA counts (16-nt to 29-nt) in this small RNA library were 60,763, out of which a total number of 38,936 sRNAs matched the LSU rRNA database, accounting for 64% of the total sRNA population present in this fraction. Small RNAs in the other three libraries, however, exhibited similar patterns of rRNA abundance and distribution, in which the levels of identified LSU reads fell within the range of 10% to 12% of the total sRNA counts in each library. The over-representation of rRNAs in the ribosomal fraction from biological replicate 1 was unlikely to be an LSU-rRNA- specific phenomenon since mapping against SSU rRNAs (small subunit rRNAs) resulted in a similar over-presentation pattern (data not shown). This observed sequencing bias might be explained by more degradation of rRNAs in this ribosomal RNA sample. However, as shown in Figure 2.5 B and Figure 2.5 C, when mapped against the tRNA database and maize cDNA database, the sRNA distribution patterns were fairly similar between the two biological replicates. In all of the four ribosomal and non-ribosomal libraries, sRNA reads that matched tRNA sequences (tRNA-sRNAs) exhibited substantial distributions in the 16-nt population. Interestingly, although within the size range of 16-nt to 20-nt the distribution patterns of tRNA-sRNAs

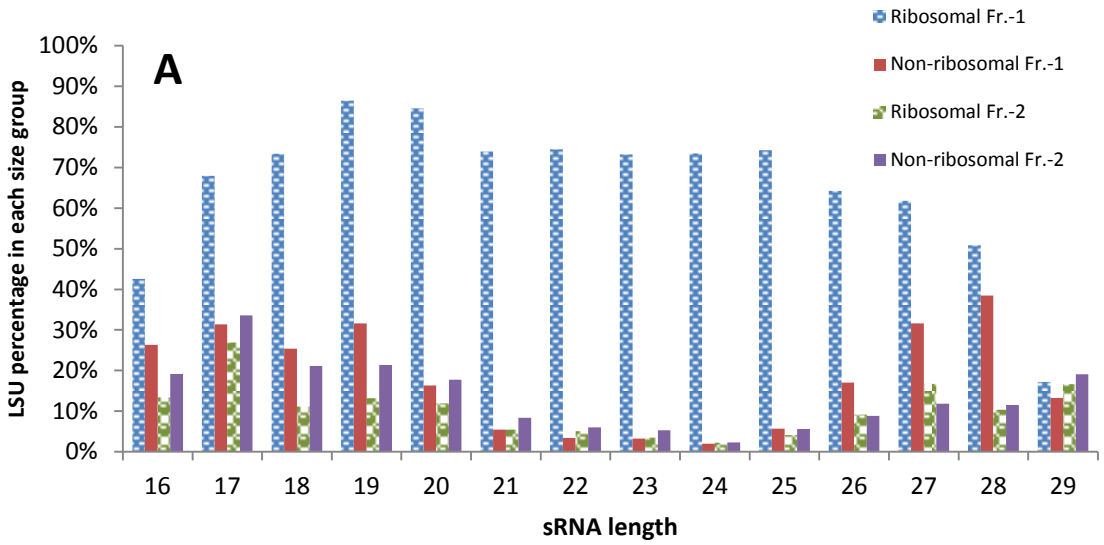
were to some degree similar across the four libraries, a clearly distinctive pattern between the ribosomal fractions and non-ribosomal fractions was observed from the 21-nt and 22-nt sRNA species. In both of the two ribosomal replicates, 21-nt and 22-nt sRNAs displayed significantly higher levels of tRNA-sRNAs than did their corresponding counterparts in the two non-ribosomal replicates. Figure 2.5 C shows the distribution patterns of sRNAs mapped to the maize cDNA database. Despite the various degrees of variance between the two replicates, a general pattern could be drawn for sRNAs within the sizes of 14 nt to 20 nt such that sRNAs matching the maize transcriptome were more abundant in ribosomal than non-ribosomal fractions. To summarize, the overall similar distribution patterns of different sRNAs supported the validity of sample preparations and library construction with the caveat that the discrepancy observed in rRNAs in biological replicate 1 (Figure 2.5.A) could reflect a faster rate of rRNA degradation.

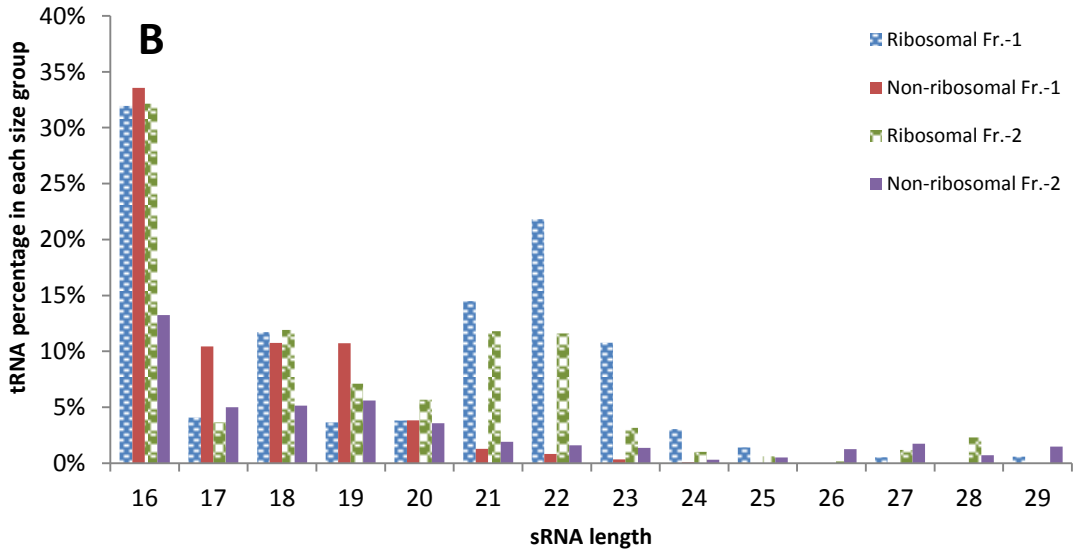
Table 2.1 Summary of the percentage of non-coding RNAs detected in individual libraries.

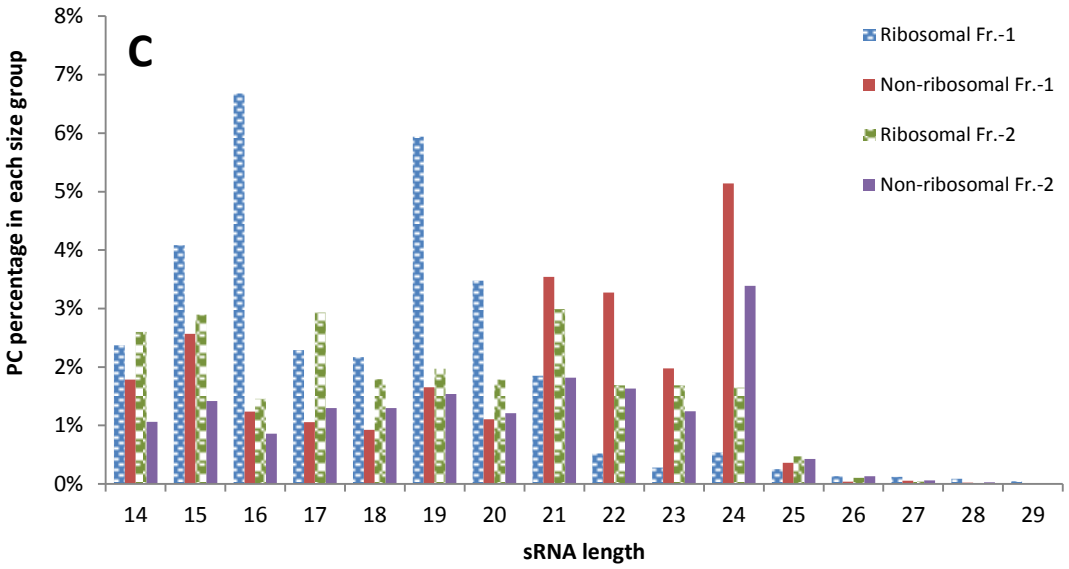
	Ribosomal Fraction-1	Non-ribosomal Fraction-1	Ribosomal Fraction-2	Non-ribosomal Fraction-2
tRNA-sRNA	16.09%	4.47%	10.16%	2.96%
rRNA-sRNA	64.08%	9.64%	10.80%	11.64%
snRNA-sRNA	0.36%	0.22%	0.37%	0.54%
snoRNA-sRNA	0.31%	0.04%	0.02%	0.03%
Other	19.16%	85.63%	78.65%	84.83%

Figure 2.5 Size distributions of sRNAs matching rRNA, tRNA and protein-coding (PC) sequences in each given sRNA class.

A.Percentage of sRNAs matching LSU (Large ubunit rRNAs) B. Percentage of sRNAs matching tRNAs C.Percentage of sRNAs matching maize cDNAs. Graphs were generated by dividing the total counts of the identified RNA by the total sRNA reads for each given length.


A
Ribosomal Fr.-1
Non-ribosomal Fr.-1
Ribosomal Fr.-2
Non-ribosomal Fr.-2
LSU percentage in each size group
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
16
17
18
19
20
21
22
23
24
25
26
27
28
29
sRNA length


B
Ribosomal Fr.-1
Non-ribosomal Fr.-1
Ribosomal Fr.-2
Non-ribosomal Fr.-2
tRNA percentage in each size group
40%
35%
30%
25%
20%
15%
10%
5%
0%
16
17
18
19
20
21
22
23
24
25
26
27
28
29
sRNA length


C
PC percentage in each size group
8%
7%
6%
5%
4%
3%
2%
1%
0%
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
sRNA length
Ribosomal Fr.-1
Non-ribosomal Fr.-1
Ribosomal Fr.-2
Non-ribosomal Fr.-2

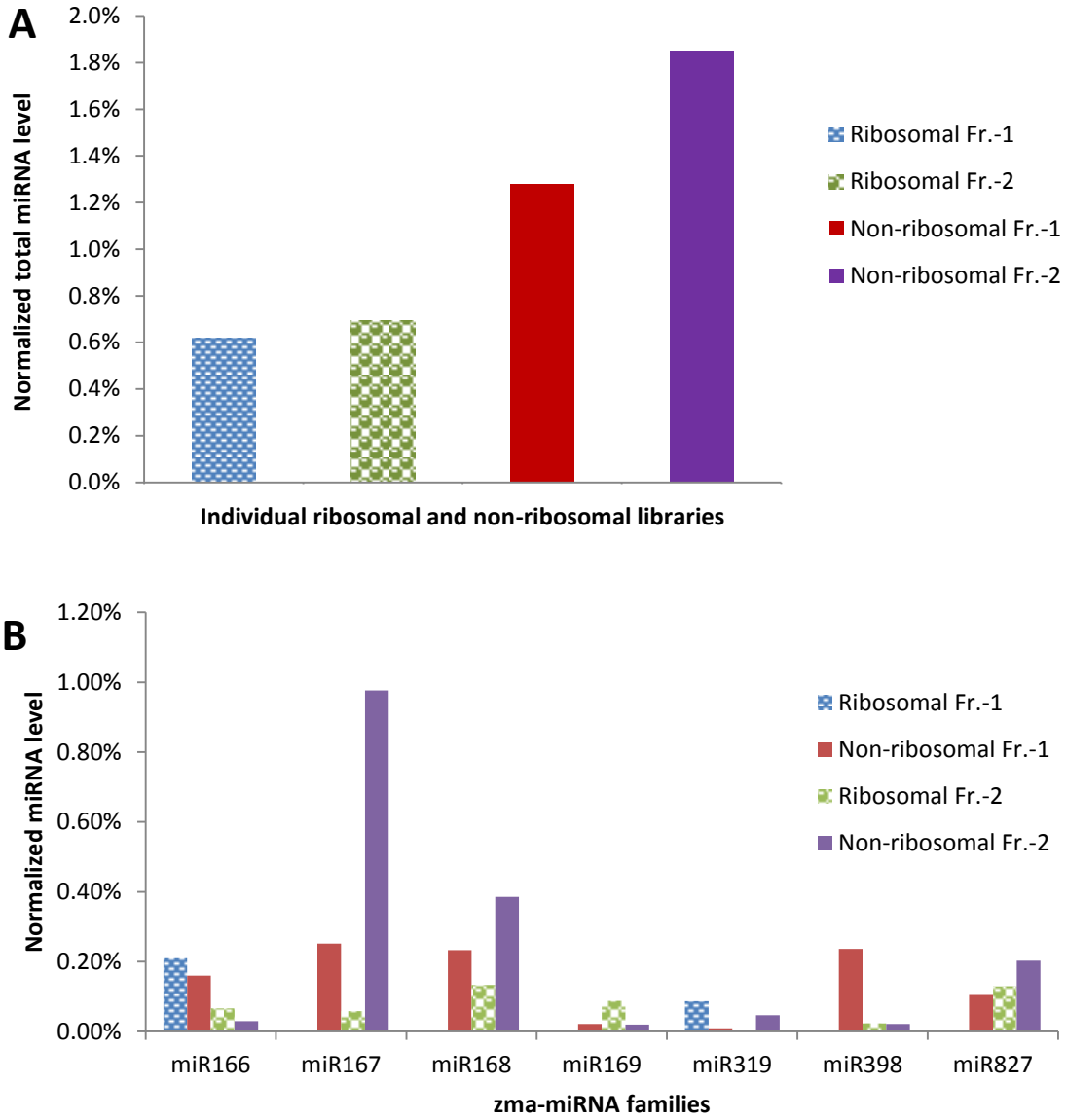


Figure 2.6 Relative abundance of zma-miRNAs identified in different libraries.

The normalized levels of miRNAs were obtained by dividing the number of miRNA counts by the number of 18–24 nt sRNA reads in each library, respectively. A. The normalized total miRNA level in 4 individual libraries. B. The normalized levels of selected miRNAs in 4 individual libraries, respectively.
The X axis indicates the conserved maize miRNA families identified. The Y axis indicates the normalized levels of miRNAs detected in this study.

Identification of conserved maize miRNAs in individual libraries

Conserved miRNA families have been found among different plant species and shown to have essential functions in various aspects of plant life from development to stress responses and hormone signaling. To date, there are 319 mature maize miRNAs and 170 miRNA precursors in the miRNA database miRBase (www.mirBase.org; Kang et al., 2012). To identify conserved miRNAs in this study, small RNA sequences were aligned to maize mature miRNAs and precursors with a maximum of one mismatch (http://ftp.maizesequence.org/current/mirna/).

A total of 18 conserved zma-miRNA families were identified in this deep sequencing study. After normalizing the expression level of each identified miRNA family by the total counts of 18-nt to 24-nt sRNAs in each library, we chose to further study the miRNA family that exhibited a relative abundance higher than 0.02% in at least one of the four libraries. As shown in Figure 2.6 A, differential distributions of miRNAs in the ribosomal and non-ribosomal fractions were observed consistently in both of the biological replicates. The normalized total miRNA levels detected in ribosomal fractions were slightly over 0.6% while in the non-ribosomal libraries, the levels were between 1.3% and 1.9% (Figure 2.6 A). Figure 2.6 B shows the distribution of selected miRNA families (families with highest relative abundance) in four individual libraries. In non-ribosomal fractions, the most prominent miRNA families were miR167 and miR168 (Figure 2.6.B). Even though the relative abundance of total miRNA populations in the ribosomal fractions was lower, there was a clear change in the most abundant miRNA families: instead of miR167 and miR168 being

the best represented miRNAs as exhibited in the non-ribosomal fractions, miRNA166, miR169, miR319 and miR827 were found to be among the more abundant miRNA families in at least one of the ribosomal replicates (Figure 2.6 B). This result is particularly interesting because the differential distribution of miRNA families in the ribosomal- and non-ribosomal fractions suggested that different miRNAs have various degrees of preference in associating with ribosomes. Not all of the miRNA families that were detected in the ribosomal fraction from biological replicate 2 were detected in biological replicate 1. This discrepancy was possibly due to the shadowing effect of the overwhelmingly high level of rRNAs (as shown in Table 2.1 and Figure 2.5 A).

It is worth noting, however, that for any given miRNA family, the levels for the ribosomal fraction and non-ribosomal fractions were not comparable in a quantitative manner due to lack of a common normalizer between these two fractions. Therefore, one can compare the abundance of different miRNA families only within the same type of library, whether it be the ribosomal or non-ribosomal fractions.

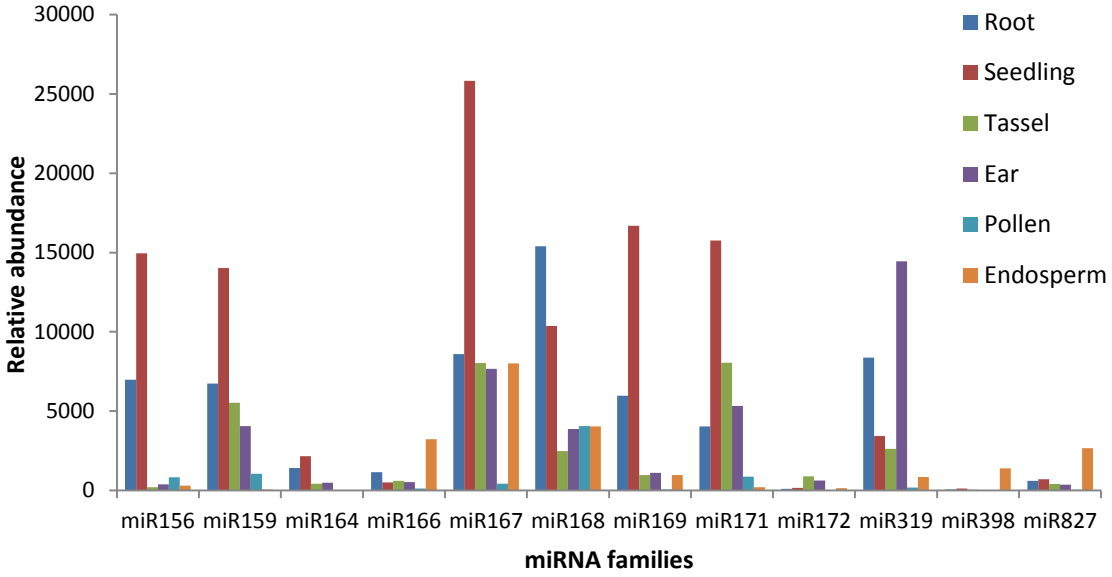


Figure 2.7 Comparison of the zma-miRNA abundance in endosperm and several other maize tissues.

The level of expression of miRNA gene families in each tissue was assessed by counting the number of 18–22 nt reads mapping to each family, normalized by the total number of 18–22 nt reads in the respective libraries. Counts are in reads per million (RPM). Small RNA data on maize root, seedling, tassel, ear and pollen were taken from Zhang et al. 2009.

When combining the data from both ribosomal and non-ribosomal fractions, we found that the miRNA results from our study were consistent with the miRNA data generated in previous studies from developing and mature seeds. Even though in endosperm their overall expression levels were relatively low, miR166, miR167, miR168 were the most abundant miRNAs (Wang et al., 2011a; Kang et al., 2012; Li et al., 2013). As shown in Figure 2.7, when compared to the Next-Gen sequencing data from different maize tissues, miR167, miR168 were almost always among the highly expressed maize miRNAs, whereas miR166 seemed to be preferentially up regulated in endosperm tissue (Zhang et al., 2006a; Zhang et

al., 2009). In addition, miR156, miR159, miR171 were among the abundant miRNAs in many maize tissues; however, in endosperm they showed very low expression levels. Interestingly, miR398 and miR827 seemed to be highly expressed in endosperm compared to other tissues tested. This observation might suggest an endosperm-specific regulatory role of these two miRNAs. In Table 2.2, I summarized the experimentally verified or computationally predicted targets of all the maize miRNAs detected in the present study.

Table 2.2 A selection of computationally predicted targets of the miRNA families detected in this study. Table was adopted from Zhang et al. 2009

	No. of Family Members	**Selected Target Prediction**
miR-167	10	Auxin response factors
miR-168	2	Argonaute and dicer protein
miR-166	14	HD-ZIP family (eg. Maize rld1 / rld2)
miR-398	2	unknown
miR-319	4	MYB domain transcription factor; DNA-binding TCP Transcription factor
miR-169	18	CCAAT-binding transcription factor NYF transcription factor
miR-156	12	SBP-box transcription factors (eg. tga1)

Table 2.2 (Continued)

miR-827	1	SPX domain protein Major facilitator superfamily
miR-408	2	Cupredoxin Multicopper oxidase
miR-171	14	GRAS transcription factor
miR-172	5	AP2-ERF transcription factor (eg. gl15)
miR-164	8	No apical meristem (NAM) protein; NAC1
miR-159	11	MYB domain transcription factor
miR-395	16	Sulphate anion transporter

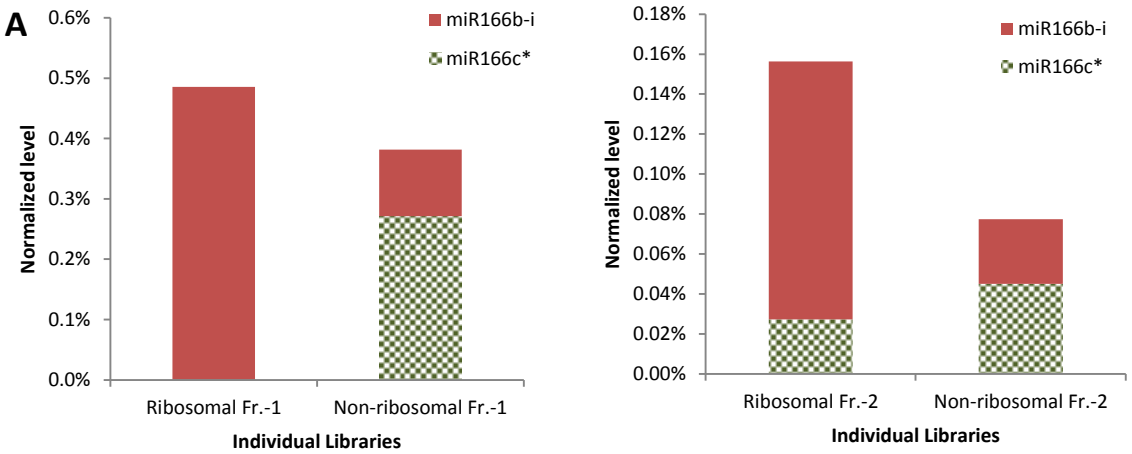
The role of miRNA*s in plant development

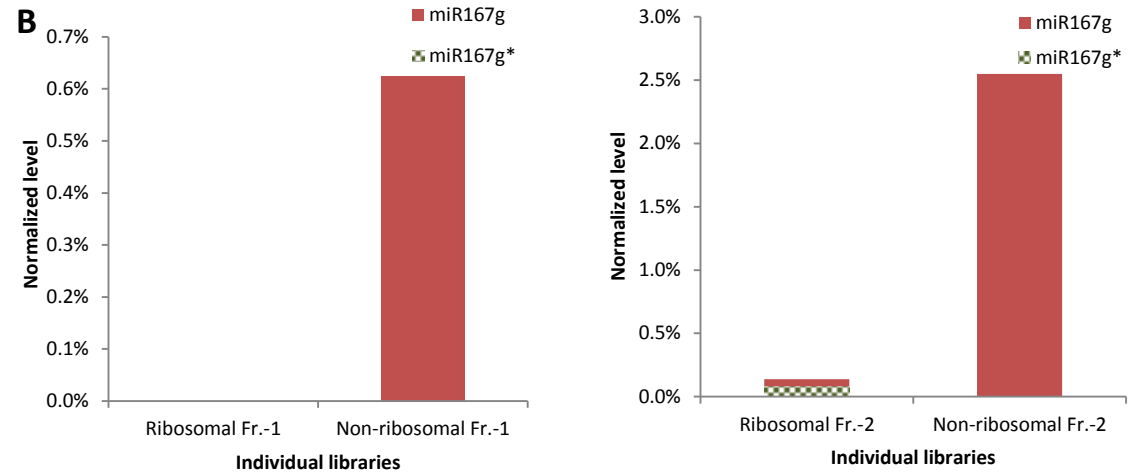
In plants, during miRNA biogenesis, miRNA:miRNA* duplexes are processed by DCL1 in a sequential manner from longer stem-loop RNA precursors. The mature miRNA strand then is selected as the functional miRNA during RISC maturation while miRNA* is believed to be degraded (Bartel, 2004). The model for selection of the mature strand was based on the thermodynamic structures of the miRNA:miRNA* duplexes (Schwarz et al., 2003). However, recent research led to the suggestion that miRNA*s could also down-regulate their target genes in both plants and animals (Rajagopalan et al., 2006; Devers et al., 2011; Yang et al., 2011a; Manavella et al., 2013). In our sequencing results, a few of the miRNA*s were

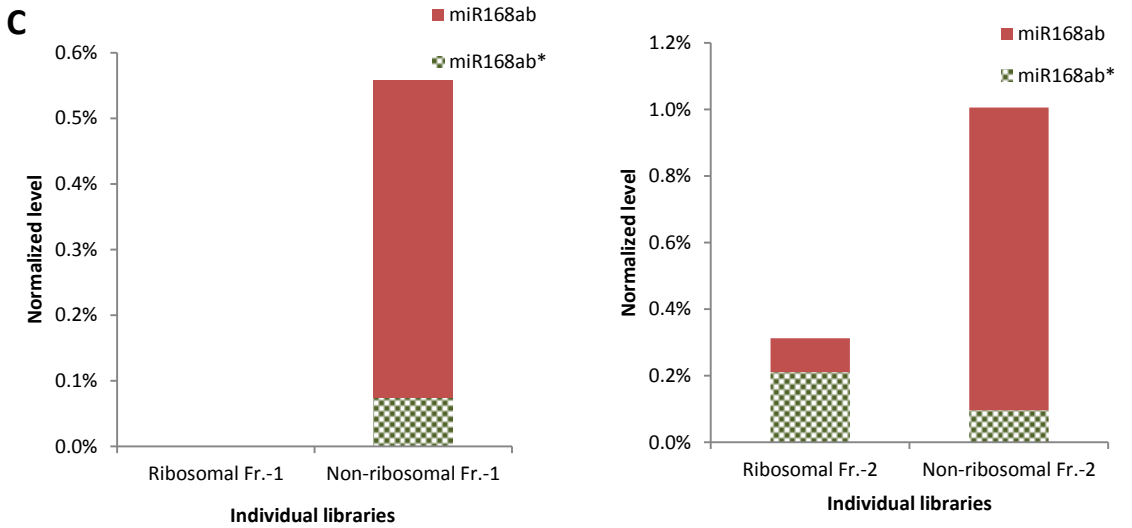
found and the relative abundance of these miRNA*s was sometimes even higher than that of their mature miRNA counterparts. As shown in Figure 2.8 D, the total reads for miR827* were much greater than those for the mature miR827 in both the ribosomal and non-ribosomal fractions. In the case of miR168, approximately 20% of the total sRNA reads matching the miR168 precursors were characterized as either miR168a* or miR168b*. Although the mature miRNA sequences of miR168a and miR168b are identical, the presence of both miR168a* and miR168b* indicated the coexpression of these two isoforms in maize endosperm. Of all the miR168* reads, approximately 70% belonged the miR168a* group while the rest were identified as miR168b*. Similar to miR167*, miR168* (which is not identical) was preferentially found in the ribosomal fraction. In contrast, miR166* was favored to reside in the non-ribosomal fraction (Figure 2.8 A). Because there is a precedence for miRNA*s functioning as mature miRNAs, it is possible that these miRNA*s could also be *de facto* miRNAs.

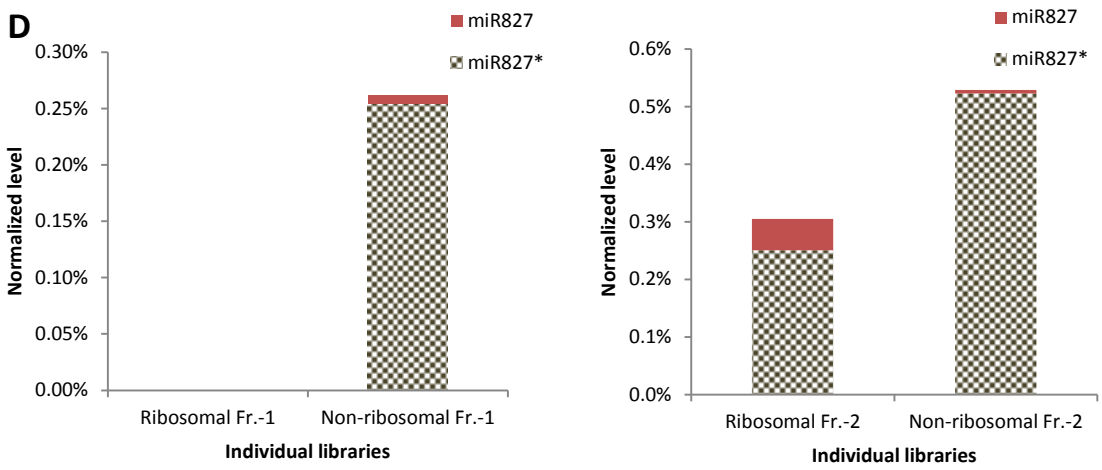
Figure 2.8 Normalized levels of miRNAs and miRNA*s detected in each library.

A. miR166 and miR166* B. miR167g and miR167g* C. miR168 and miR168* D. miR827 and miR827*
Graphs were generated by dividing the counts of each miRNA species by the total 20-nt to 22-nt sRNA counts in each individual library.


A
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
Normalized level
miR166b-i
miR166c*
Ribosomal Fr.-1
Non-ribosomal Fr.-1
Individual Libraries
0.18%
0.16%
0.14%
0.12%
0.10%
0.08%
0.06%
0.04%
0.02%
0.00%
Normalized level
miR166b-i
miR166c*
Ribosomal Fr.-2
Non-ribosomal Fr.-2
Individual Libraries

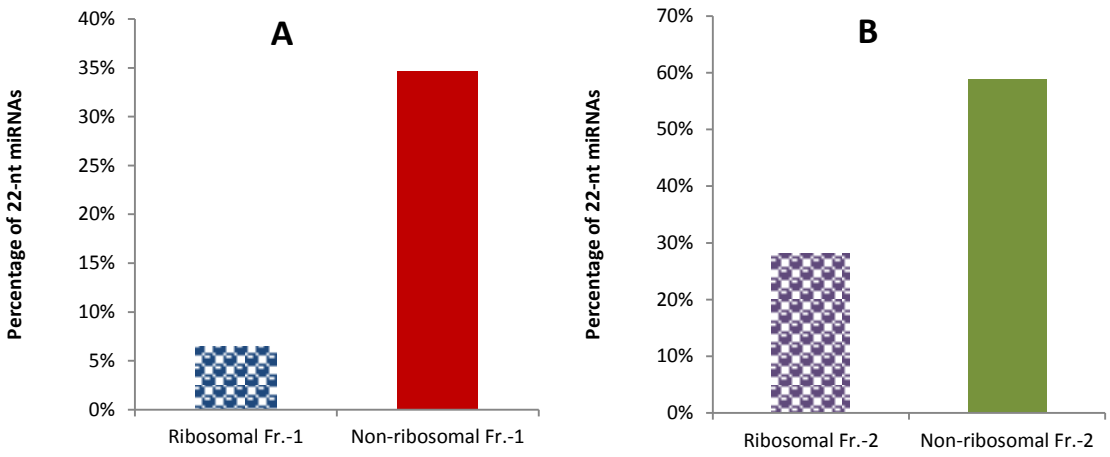

B
0.7%
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
Normalized level
miR167g
miR167g*
Ribosomal Fr.-1
Non-ribosomal Fr.-1
Individual libraries
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
Normalized level
miR167g
miR167g*
Ribosomal Fr.-2
Non-ribosomal Fr.-2
Individual libraries


C
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
Normalized level
miR168ab
miR168ab*
Ribosomal Fr.-1
Non-ribosomal Fr.-1
Individual libraries
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
Normalized level
miR168ab
miR168ab*
Ribosomal Fr.-2
Non-ribosomal Fr.-2
Individual libraries



Potential tasiRNA-triggering miRNAs

Further analyses on the size distribution of miRNAs identified in this study revealed very distinctive patterns of distribution of 21-nt and 22-nt miRNAs in the ribosomal and non-ribosomal fractions, respectively. As shown in Figure 2.9, in each of the two biological replicates, the percentage of 22-nt miRNAs in non-ribosomal fractions was significantly higher than it was in ribosomal fractions.



	21-nt	22-nt
Ribosomal Fr.-1	115	8
Non-ribosomal Fr.-1	192	102

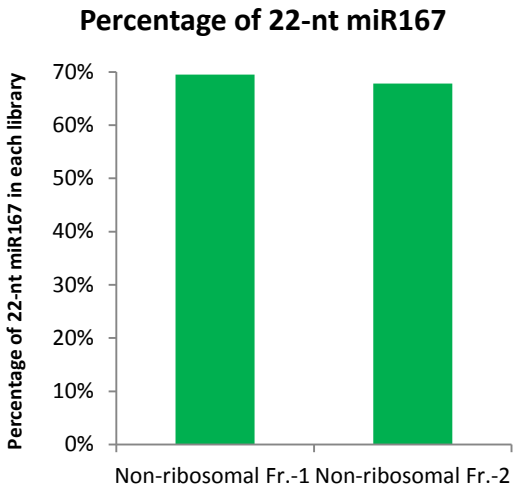
	21-nt	22-nt
Ribosomal Fr.-2	136	53
Non-ribosomal Fr.-2	246	350

Figure 2.9 Percentage of 22-nt miRNA in ribosomal and non-ribsomal fractions

Panels A and B: the percentage of 22-nt miRNA in biological replicate 1and 2
The graphs represent the percentage of 22-nt miRNAs in each library. Graphs were generated by dividing the number of 22-nt miRNAs by the total number of 21-nt and 22-nt miRNAs.

One example of the co-presence of 21-nt and 22-nt isomers within the same miRNA family came from miR167. As shown in Figure 2.10, the non-ribosomal miR167 populations can be further divided into two groups based on their size differences. Approximately 70% of the miR167 population detected in both of the two biological replicates was the 22-nt species. This finding raises the question as to whether or not this 22-nt miR167g can trigger the production of a group of secondary siRNAs, namely trans-acting siRNA (tasiRNA), as previous studies have shown that 22-nt miRNAs bear a unique functionality in triggering

tasiRNA biogenesis rather than directly targeting the cognate protein-coding mRNAs for degradation (Chen et al., 2010; Cuperus et al., 2010; Manavella et al., 2012).



	Non-ribosomal miR167-Rep1	Non-ribosomal miR167-Rep2
miR167 22-nt	57	268
miR167 21-nt and smaller	25	127

Figure 2.10 Percentage of 22-nt miR167 in non-ribosomal RNA fractions

Results from two biological replicates are shown here. The graph was generated by dividing the number of 22-nt miRNA167 counts by the number of total miR167 counts in each library.

To investigate the potential for the 22-nt miR167g in triggering the production of tasiRNAs, we further analyzed the structure of the stem-loop precursor of miR167g (pre-miR167g). As shown in Figure 2.11, a bulge was present in the middle region of the miR:miR* duplex in pre-miR167g, contributing to the 1-nt size difference between the miR167g mature sequence (pink region in Figure 2.11) and the miR167g* (blue region in Figure 2.11). To date, the majority of miR167g sequences deposited in miRbase (http://www.mirbase.org/) were in the form of 21 nt. The finding in our study that nearly 70% of the miR167g population took the form of 22-nt was in agreement with the previous report by Manavella et al., in which they showed that the asymmetrically positioned bulges in the miRNA:miRNA* duplexes triggered the production of secondary siRNAs, which in turn silenced other genes *in trans* (Manavella et al., 2012*)*. Interestingly, so far the majority of tasiRNAs that have been experimentally verified derived from non-protein-coding RNA precursors (Montgomery et al., 2008a; Montgomery et al., 2008b; Felippes and Weigel, 2009; Marin et al., 2010). Therefore, the fact that the 22-nt miR167g was found only in the non-ribosomal fraction indirectly supported its potential role in directing the production of tasiRNAs.

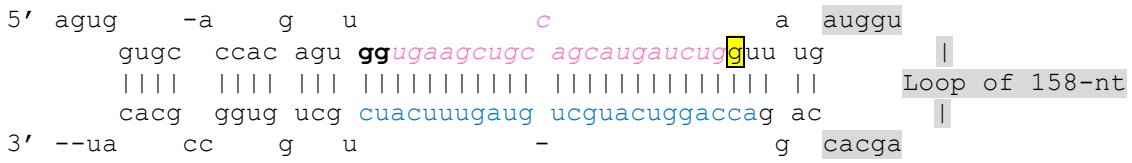
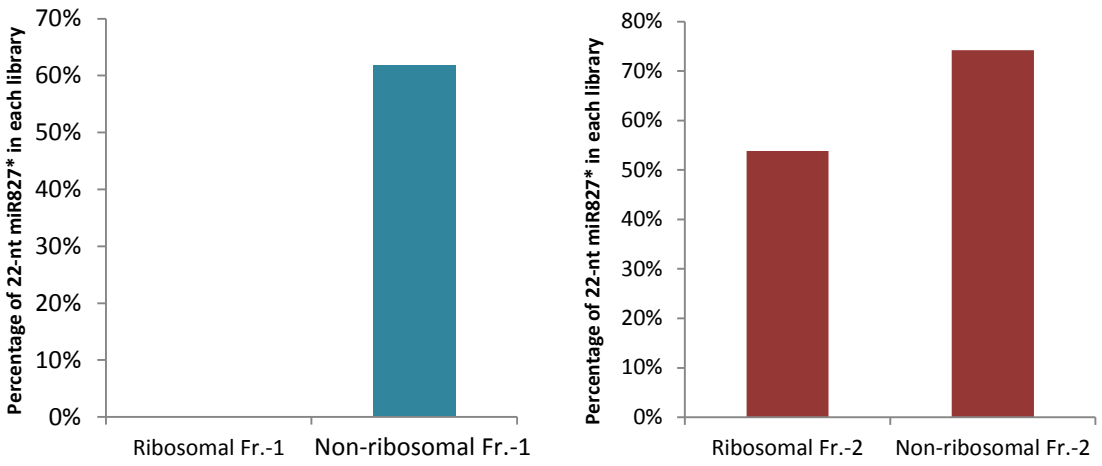


Figure 2.11 Structure of zma-pre-miR167g.

Blue region: miR167g*; Pink italic region: 21-nt miR167g (from miRbase.org) The boxed "g" is the additional nucleotide found on the 22-nt miR167g

Similar to the observation made for miR167, the miR827* family was also composed of two major size groups with one being 21-nt and smaller, and the other one being 22-nt (Figure 2.12). As shown in Figure 2.12, around 60% of the miR827* populations detected in both the ribosomal and non-ribosomal fractions were 22-nt. Even though miR827* was not detected in the ribosomal fraction from biological replicate 1, the non-ribosomal results from that biological replicate recapitulated the results from biological replicate 2.



	miR827* 22-nt	miR827* 21-nt and smaller		miR827* 22-nt	miR827* 21-nt and smaller
Ribosomal Fr.-1	0	0	Ribosomal Fr.-2	42	36
Non-ribosomal Fr.-1	21	13	Non-ribosomal Fr.-2	49	33

Figure 2.12 Percentage of 22-nt miRNA827* in ribosomal and non-ribosomal fractions

Graphs were generated by dividing the number of 22- miRNA827* by the number of total miR827* counts in each library.

Discussion

The maize endosperm makes up to 80% of the mature kernel weight and is the predominant sink for carbon assimilates during reproductive growth (Lur and Setter, 1993; Schweizer et

al., 1995). Elucidating the cellular and molecular events that contribute to normal endosperm development will provide a better knowledge base to understand the regulatory components of grain qualitative and quantitative traits that might be manipulated to generate maize lines with more desirable traits. The endosperm nourishes the developing embryo and is essential for proper development.

Endosperm usually displays decreased levels of DNA methylation compared to the embryo or vegetative tissues (Lauria et al., 2004; Gehring et al., 2009; Hsieh et al., 2009b). In Arabidopsis, this hypomethylation is established by both the transcriptional repression of the maintenance DNA-methyltransferase MET1 (Jullien et al., 2008) and the active DNA demethylation by the DNA glycosylase DEMETER (DME; Choi et al., 2002; Hsieh et al., 2009) . However, the functional significance of this genome-wide demethylation of the endosperm is yet to be understood. Because the endosperm genome is not transmitted to the progeny, the transient activation of transposons carries a relatively low cost. Transposon activation and siRNA accumulation might in fact contribute to enhanced levels of DNA methylation in the egg cell (and later the embryo) via siRNA transport (Hsieh et al., 2009b). Indeed, this notion was supported by the observation that 24nt siRNAs accumulated in the female gametophyte and in the endosperm (Mosher et al., 2009).

The sRNA length distribution of the non-ribosomal sRNAs in the present study resembled what has been reported from other deep sequencing studies conducted in maize and various organisms including Arabidopsis, with 24-nt sRNAs being the most prominent species

(Rajagopalan et al., 2006; Morin et al., 2008; Szittya et al., 2008; Wang et al., 2011a; Ding et al., 2012; Li et al., 2013). In the non-ribosomal fractions, the sRNA size distributions were consistent with previous deep sequencing studies in maize seeds (Wang et al., 2011a; Kang et al., 2012) and other organisms such as *Medicago truncatula* (Szittya et al., 2008), rice (Morin et al., 2008), peanut (Zhao et al., 2010) and Arabidposis (Rajagopalan et al., 2006). The finding of 24mers being the most abundant sRNA population in the non-ribosomal fractions was suggestive that the heterochromatic endogenous siRNAs, which frequently assume the same signature form as 24mers, play a robust role during maize endosperm development, possibly via genomic imprinting and RNA silencing (Springer, 2009; Mosher and Melnyk, 2010; Lu et al., 2012; Migicovsky and Kovalchuk, 2012). This finding was also in agreement with the notion that the 24-nt heterochromatic endogenous siRNAs were active in endosperm tissue due to the hypomethylated status in many siRNA-generating areas such as transposons and repetitive regions (Hsieh et al., 2009b; Hsieh et al., 2011). Interestingly, studies in wheat show a different trend in the small RNA size distribution, with the sequenced reads peaking at 21nt (Yao et al., 2007; Wei et al., 2009). Unlike pine trees which lack the DCL3 enzyme responsible for the 24-nt siRNA production, wheat does have DCL3 activity as the wheat germ extract used in studies *in vitro* was able to dice long dsRNA precursors into 24-nt siRNAs (Tang et al., 2003). Thus, the altered size distribution indicates that even among monocots, shifted preference in DCL usage in producing small RNAs might be present.

In contrast to the 24-nt sRNAs that dominated the sRNA populations in the non-ribosomal fractions, sRNAs in ribosomal fractions exhibited a distinctively different pattern of size distribution. First of all, the relative abundance for sRNA species smaller than 20 nt was

dramatically elevated in the ribosomal fractions. A prominent peak was observed at 16-nt in ribosomal fractions. Further sequence analyses revealed the major components of this peak to be tRNAs (approximately 30% in both replicates) and rRNAs (approximately 40% and 10% in two replicates, respectively). Since both the 16-nt tRNAs and rRNAs were potentially the degradation products from intact RNAs, they might serve as good indicators to estimate the degree of RNA degradation that had occurred in each RNA sample. Secondly, the major discrepancy in this current deep sequencing study came from the abnormally high levels of sRNAs matching rRNAs in the ribosomal fraction from biological replicate 1. As shown in Figure 2.5 A, for each of the analyzed sRNA size species, a substantial amount of rRNA fragments was present in this sample. This over-representation of rRNA fragments likely was caused by an unknown factor that caused rapid rRNA degradation. Consequently, the over-representation of the rRNA fragments in this library would have introduced a shadowing effect during deep sequencing that would mask the detection of many ribosome-associating miRNA families.

Because in this study we aimed to understand the behavior of regulatory sRNAs such as miRNAs and siRNAs, sRNA species within the size of 21 nt to 24 nt were the major sRNA populations of interest. When comparing the length distribution of ribosomal- and non-ribosomal sRNA populations, interestingly, we found that instead of the 24-nt sRNAs being the predominant species, in the ribosomal samples the 21-nt sRNAs were the most abundant ones. In plants, the 24-nt heterochromatic endogenous siRNAs preferentially associate with AGO4 and are known to be involved in chromatin remodeling and transcriptional silencing

in nuclei; consequently, they are unlikely to be interacting with the translation machinery (Henderson and Jacobsen, 2007; Chan, 2008; Matzke et al., 2009). Our finding that 24-nt sRNAs were preferentially distributed in the non-ribosomal fractions indeed was in line with the absence of the 24-nt siRNAs in the RNAs associated with the translation machinery.

The differences in miRNA abundance between this study and previously published data on developing maize kernels could have resulted from the different ways of sample preparation: In the previous study, sRNAs from developing kernels (embryo and endosperm) at different stages (10, 15, 20, 25, and 30 DAP) were pooled and treated as one RNA library, while in my study, sRNAs from only 18DAP endosperm were analyzed. In addition, when cross referencing data from various maize small RNA studies including immature and germinating seeds, I found that maize embryos from dry and germinating kernels exhibited miRNA levels between 10% and 15% of the total counts of sRNAs ranging from 18-nt to 30-nt (Li et al., 2013) while maize developing seeds had a miRNA level at approximately 1/3 of that found in the germinating seeds (Wang et al., 2011a; Kang et al., 2012; Zhai et al., 2013). In this present study, both the non-ribosomal and ribosomal miRNA levels were below 2%. This finding suggests that in 18DAP endosperm, the overall expression level of miRNAs is relatively low, and because the mature maize seeds contain a much higher level of miRNAs compared to the developing immature seeds (Wang et al., 2011a; Kang et al., 2012; Zhai et al., 2013), it is likely that miRNAs are involved more actively during maize germination than in the seed maturation processes.

In a previous study, miR319 and miR169o were shown to be present in maize seeds but not mature leaves (Kang et al., 2012). Consistently in present study, miR319 and miR169o were detected and both of them displayed preferential distributions with ribosomal-associating RNAs. MiR319 was one of the earliest miRNAs that was identified and characterized, owing to the prominent jagged leaf phenotypes of the Arabidopsis *miR319*/*jaw* mutants (Palatnik et al., 2003). In Arabidopsis, miRNA319 regulates the expression levels of five members in the TCP transcription factor family (Palatnik et al., 2003; Nag et al., 2009), and in maize it was predicted to target TCPs as well as several MYB transcription factors (Zhang et al., 2009). The best documented role of miR169 is its response to nitrogen starvation (Liu et al., 2008; Shukla et al., 2008; Ruiz-Ferrer and Voinnet, 2009). In Arabidopsis, miR169 targets the NFY transcription family and is involved in various abiotic stress responses (Li et al., 2008; Pant et al., 2009; Liang et al., 2012). In addition to miR169 and miR319, miR166 and miR827 also exhibited disproportionally high levels of distribution in the ribosomal fraction in my study. In maize, Timmermans et al. reported that miR166 targets two members of the HD-ZIP transcription family, namely Rolled leaf 1 and Rolled leaf 2 (Rld1/2). Both of Rld1 and Rld2 are involved in the establishment of leaf polarity and the normal leaf development (Juarez et al., 2004). It has been suggested that in maize seeds, miR166 and its targets enhance potential lateral organ and meristem formation during seed germination (Li et al., 2013). However, the role of miR166 in endosperm remains elusive.

Computational prediction shows that in maize, miR167 targets auxin response factors (ARFs) while miR168 targets AGO1. These data suggests that regulating auxin sensing and response

pathways, as well as miRNA/RISC homeostasis are important during endosperm development. In Arabidopsis, miR398 targets two genes encoding copper/zinc superoxide dismutases (CSDs) and a copper chaperone (CCS1) responsible for delivering copper to these CSDs (Sunkar et al., 2006; Beauclair et al., 2010). Interestingly, in maize, one of the targets of zma-miR398 was predicted to be the maize homolog of TRANSPARENT TESTA 1 (TT1; Maize GDB ID: GRMZM2G068710_T07; Dai and Zhao, 2011) . In Arabidopsis, *TT1* gene expression was detected in developing ovules and young seeds only (Sagasser et al., 2002), and the gene was shown to be responsible for controlling flavonoid accumulation in endothelium cells during Arabidopsis seed development (Sagasser et al., 2002; Appelhagen et al., 2011). MiR827 is a relatively less conserved miRNA family. Interestingly, miR827 was found to be expressed at a much higher level in endosperm than in any other tested maize tissue (Figure 2.6). In Arabidopsis and several other organisms, it has been shown to be involved in regulating nitrate/phosphate homeostasis (Lin et al., 2010; Kant et al., 2011; Liang et al., 2012). In rice, miR827 has been shown to regulate two genes encoding endosperm SPX-MFS proteins in response to phosphate starvation (Lin et al., 2010). Since in the endosperm a great amount of storage proteins is being synthesized, it is reasonable to speculate that the high level of miR827 in endosperm indeed reflects its essential role in sustaining normal nitrogen and amino acid metabolism during monocot endosperm development. Figure 2.13 shows the potential miRNA regulatory network in maize.

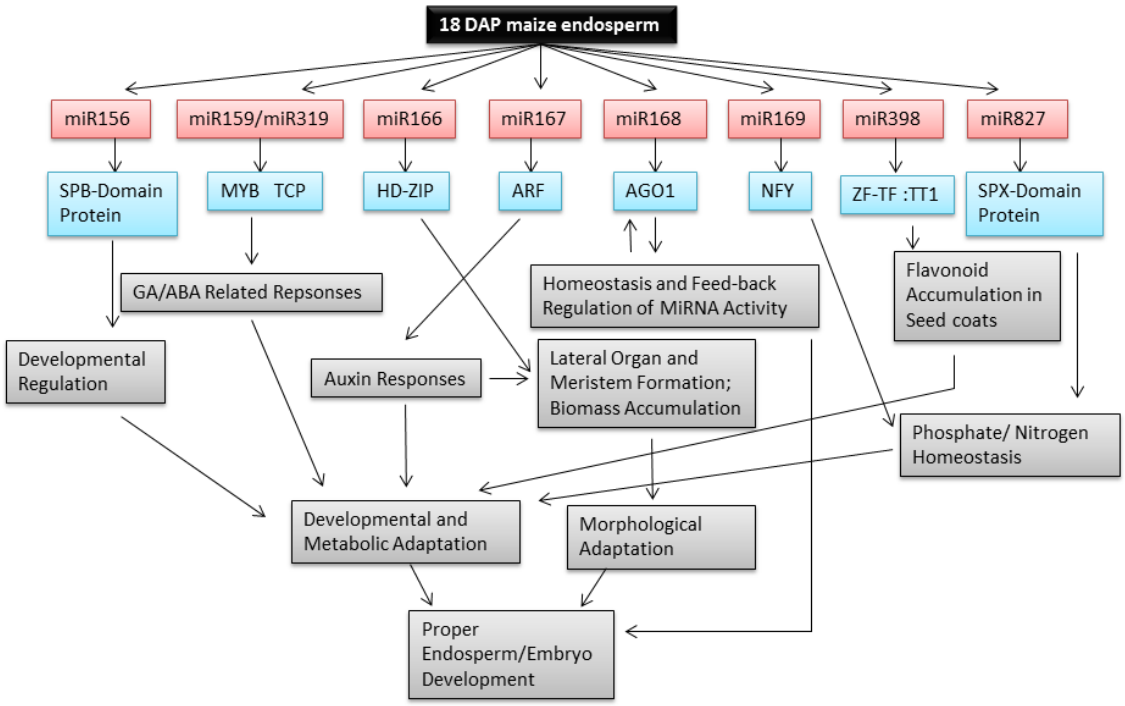


Figure 2.13 The potential regulatory network for miRNAs in maize endosperm.

miRNAs shown in red boxes represent the more abundant miRNA families detected in this deep sequencing study. Potential miRNA targets in maize are shown in the blue boxes. Biological processes potentailly regulated by various miRNAs are shown in grey boxes.

As shown in Figure 2.8 B, even though no miRNA167g was detected in the ribosomal fraction from biological replicate 1, it was consistent between the two biological replicates that miR167g was detected in non-ribosomal fractions. In contrast to miR166, miR167* was predominantly found to be associated with the ribosomal RNA fraction. However, the portion of miR167* in the overall miR167 population was very small (5%), thus it would be difficult to discriminate it as a signal above noise. Intriguingly, the same phenomenon observed in

miR167 was observed in the case of miR168. The majority of miR168* was found in the ribosomal fraction (Figure 2.8 C). Computational analyses identified a gene encoding the maize ubiquitin-1 homolog (Maize GDB ID: GRMZM2G170843_T01) to be one of the miR168* putative targets (Dai and Zhao, 2011).

In the case of miR166, as described earlier, the majority of miR166* was localized in the non-ribosomal fraction (Figure 2.8.A). Several maize genes were predicted to be the potential miR166* targets (Dai and Zhao, 2011), among which were the leucine-rich-repeat receptor-like kinase (Maize GDB ID: GRMZM2G048801_T01), the flavin containing amine oxidoreductase-like protein (Maize GDB ID: GRMZM2G137820_T02), and the maize polynucleotidyl transferase /UTP-glucose-P-uridyltransferase homolog protein (Maize GDB ID: GRMZM2G134903_T01). On the one hand, it seems plausible that the reason a large population of miR166* was found in the non-ribosomal RNA fractions was due to the release of the miR166 passenger strands during the maturation of RISC complexes in the cytoplasm. On the other hand, the extremely high percentage of miR166* in the overall non-ribosomal miRNA166 population made it rather difficult to simply regard it as the "degradation product". Indeed, previous research has demonstrated that miRNA*s might act just as mature miRNAs in regulating target expression (Devers et al., 2011; Zhang et al., 2011b; Manavella et al., 2013). And very often the biological functionality of these miRNA*s is reflected by their relatively high abundance in the corresponding deep sequencing data (Manavella et al., 2013). To experimentally verify the notion that miR166* in maize endosperm acts as a *bona fide* functional miRNA, a PARE (Parallel Analysis of RNA Ends) data from maize

endosperm tissue needs to be constructed. Reads matching the predicted cleavage site (between the 10^{th} and 11^{th} nt of miRNAs) could be treated as the potential degradation products from the miRNA-mediated cleavage events.

Remarkably, in the case of miRNA827, more than 80% of the miR827 sRNA population matched the miR827* sequence. It is worth noting that even though miR827 is present in many organisms such as sugar maple (*Saccharum ssp.),* apple (*Malus domestica*), cocoa (*Theobroma cacao*), cotton (*Gossypium hirsutum*), populus (*Populus trichocarpa*) and Arabidopsis, it is still considered one of the evolutionarily "young" miRNAs (miRbase.org). As of now the miR827 allele has not been identified in any forward genetic study. Preponderant evidence from deep sequencing data, however, suggested that miR827 is involved in maintaining nitrate/phosphate homeostasis (Lin et al., 2010; Kant et al., 2011; Liang et al., 2012). By definition, miRNA* (passenger strand) is the strand from the miRNA precursor duplex that is not selected by the RISC complex. Consequently, conventionally it is believed that due to the lack of protection by the RISC core, the exposed miRNA* strands are susceptible to rapid degradation by RNA exonucleases. However, at least in maize endosperm, miR827* seems to be the functional product of miR827 biogenesis because the level of miR827 is much lower than that of miR827*. Manavella et al. reported that in a tissue-specific manner, miR170*/miR171* triggered the silencing of SU(VAR)3-9 HOMOLOG8, a new target that was recently acquired in Arabidopsis lineages (Manavella et al., 2013). Moreover, by showing that miR171* exhibited several features of canonical miRNAs such as a 5' uracil and being enriched in AGO1 immunoprecipitates, they argued

that miR170*/miR171* were *de facto* functional miRNAs (Manavella et al., 2013). Similarly, as shown in Figure 2.14, miR827* also has a 5' uracil. Aligning the miR827* back to the maize genome database (version 5.1b, B73+, maizegdb.org) resulted in only 1 hit in the entire genome, thus the miR827* reads identified were likely to be the true outcome of miRNA biogenesis rather than random RNA degradation. The plant miRNA target prediction program predicted several maize transcripts to be the targets of miR827* (Dai and Zhao, 2011), among which were RNase D family proteins, putative serine peptide S28 family proteins, and a MYB/SANT-like transcription factor.

In Arabidopsis, the two miR168 loci, namely miR168a and miR168b, produce both 21-nt and 22-nt miR168 species. While both the 21-nt and 22-nt miR168 contribute to the basal level of AGO1 mRNA regulation, the preferential stabilization of 21-nt miR168 by the AGO1 protein potentially provides another layer of flexibility in response to endogenous and environmental fluctuations (Vaucheret, 2009). More recently, Manavella et al. provided evidence in Arabidopsis demonstrating that the tasiRNA transitivity can be triggered as long as one strand of the miRNA/miRNA* duplex is 22-nt. Moreover, they showed that the asymmetrically positioned bulged bases in the miRNA/miRNA* duplex were sufficient to initiate tasiRNA production, regardless of the lengths of miRNA and miRNA* (Manavella et al., 2012). Since they could not find a case where the 22-nt strand out of the miRNA/miRNA* duplex was not caused by the presence of an asymmetric bulge within the duplex structure, they couldn't rule out the possibility that the miRNA or miRNA* size alone was sufficient to trigger tasiRNA production. In the case of maize 22-nt miR827*, however,

the pre-miR827 does not contain any bulge in the duplex area (shown in Figure 2.14). It would be very interesting if, by pairing small RNA deep sequencing data and the corresponding PARE data, one could show that tasiRNAs spawned from the miR827* directed cleavage events. If so, these findings would suggest that the miRNA lengths alone (22-nt), rather than the structures of the miR:miR* duplexes, are sufficient to trigger the tasiRNA transitivity *in vivo*.

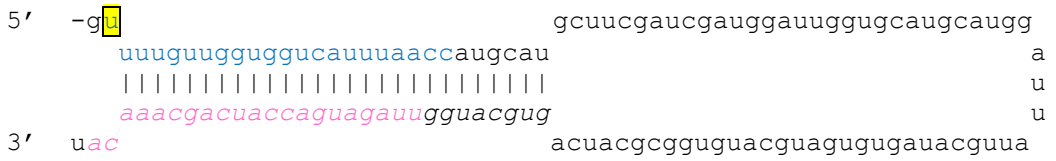


Figure 2.14 Structure of zma-pre-miR827.

Blue region: miR827*; Pink itatlic region: miR827 (from miRbase.org) The yellow boxed "u" is the additional nucleotide found on the 22-nt miR827*

Twenty-two-nt miR167 also has the potential to target one or several mRNAs, causing mRNA cleavage and/or spawning the production of a series of phased siRNAs which in turn could be incorporated into RISC to direct *in trans* cleavage/degradation of their cognate targets. Each of the tasiRNA spawned from the initial miR167-directed cleavage event has the potential to target an array of completely different mRNA targets. Because the overall

miRNA expression in endosperm is relatively low compared to other maize tissues, maize might rely on this particular mechanism to broaden the spectrum and strength of the regulation of targeted genes during endosperm development.

Materials and Methods

Plants and plant growth conditions

The maize inbred B73 was grown and self-pollinated at the Central Crops Research Station, Clayton, North Carolina, during summer field seasons. Kernels from well-filled ears were harvested at 18 day after pollination between 7:30 am and 9:00 am, immediately frozen in liquid nitrogen, and stored at -80 °C until use.

RNA isolation and cloning of maize small RNA

Maize (*Zea mays*) inbred line B73 was used in this study. Endosperm from kernels harvested at 18DAP was used for this study. The procedure used to isolate ribosome-associating RNAs was essentially the same as described previously (Zanetti et al., 2005; Mustroph et al., 2009). In summary, dissected maize endosperm were homogenized in Polysome Extraction Buffer (PEB) at a ratio of 1:2 (w/v), using a POLYTRON homogenizer. The homogenized tissue was incubated on ice for 10 min and subjected to centrifugation at 16,000xg at 4 ℃ for 15 min. The supernatant was filtered through 4 layers of Miracloth (Andwin Scientific,

Schaumburg, IL, USA) to get rid of all the remaining cell debris and then loaded onto a 2M sucrose pad and subjected to centrifugation for 3 hrs at 46,100rpm (Beckman SW50.1 rotor) at 4℃. After the ultracentrifugation the upper phase was used for extraction of the RNA from the non-ribosomal fraction, the pellet was re-suspended in resuspension buffer and used for extraction of the RNA from the ribosomal fraction. Total RNA from ribosomal and non-ribosomal fractions was isolated using a TRizol LS kit (Life Technologies, Carlsbad, CA, USA). Small RNAs were enriched by poly-ethylene glycol precipitation, separated through a 15% denaturing polyacrylamide gel, and visualized by Gel-star (Lonza, Long Beach, CA, USA) staining. Small RNAs between the size of 10-30 nt were gel-purified. Small RNAs were ligated to a 5′adaptor and 3 ′adaptor sequentially, amplified by reverse-transcription polymerase chain reaction (RT-PCR), tagged with barcodes, and used for sequencing directly (miRCat-33^{TM} miRNA cloning kit, IDT, San Jose, CA, USA; Lu et al., 2007). Sequencing was performed on a Roche 454 instrument.

Identification of conserved zma-miRNAs

After the removal of the adaptor sequences, the remaining reads longer than 18 nt were used for further analyses. First, rRNA, tRNA, snRNA, and snoRNA were removed from the small RNA sequences and the remaining sequences were compared against the maize miRNA database (http://ftp.maizesequence.org/current/mirna/) to identify conserved miRNAs. Only those small RNAs whose mature and precursor sequences matched known maize miRNAs with at most 1 mistmatch were considered to be conserved miRNAs.

Data sets

Genome sequences of *Zea mays* were obtained from Maize GDB (B73 RefGen_v2, release 5b.60, ZmB73_RefGen_v2.masked.tar.gz). Sequences of rRNAs, tRNAs, snRNAs and snoRNAs were downloaded from databases including the SILVA rRNA database (LSU database: ftp://ftp.arb silva.de/release_111/Exports/LSURef_111_tax_silva_trunc_.fasta.tgz SSU database: ftp://ftp.arb silva.de/release_111/Exports/SSURef_111_tax_silva_trunc_.fasta.tgz, for rRNA), the genomic tRNA database (http://gtrnadb.ucsc.edu/download.html -> eukaryotic-tRNAs.fa.gz, for tRNA) and non-coding RNA database (ftp://ftp.sanger.ac.uk/pub/databases/Rfam/11.0/ -> Rfam.fasta.gz, for snRNAs and snoRNAs). Annotated maize stem–loop sequences were obtained from Maize GDB (http://ftp.maizesequence.org/current/mirna/ -> ZmB73_miRNA.fasta)

Analysis of sequencing data

A quality filter of average quality score over 20 was applied to the raw reads generated by 454 sequencing. Reads with a quality score lower than 20 were discarded. Sequences were subsequently filtered by the 5' adaptor and sorted by the 10-bp distinct barcode embedded in the 5' adaptor region to generate a total of 4 libraries. After the removal of both 5' and 3' adaptor using prinseq (Schmieder and Edwards, 2011), sequences were used for subsequent alignments.

Small RNA reads matching mRNA, rRNA, siRNA, tRNA, snoRNA, snRNA were identified by aligning sRNA reads using blastn-short (BLAST+) to each individual database (for specific databases used, refer to the "data sets" section). The scoring scheme was adjusted such that the first gap was not penalized. The error score was adjusted for short reads. The BLAST result was then filtered to allow reads with no more than one mismatch or gap to be used for subsequent data analyses.

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Chapter 3

Profiling MiRNA Expression during Seed Development and Germination

Abstract

In the previous chapter, we identified a group of microRNAs that were preferentially associated with the translational machinery by doing a Next-Gen sequencing study. In this present work, we firstly sought to verify the deep sequencing data by miRNA RT-qPCR. Then we performed miRNA RT-qPCR assays to profile miRNA expression at different developmental and germinative stages from both maize endosperm and embryos. Our data suggested that (1) miRNAs were robustly involved in modulating gene expression to as adaptations to various developmental and physiological conditions during maize kernel development and germination, and (2) some of these miRNA-imposed regulations may occur co-translationally. To understand mechanistically how plant miRNAs modulate target translation, we exploited an Arabidopsis transgenic system expressing a FLAG-tagged ribosomal protein RPL18. By performing immunoprecipitation studies, we sought to establish the interaction *in vivo* between ribosomes and miRISCs. Our data suggested that indeed miRISCs were associated with ribosomes *in vivo*. Moreover, various immunoprecipitation experiments using an anti-AGO1 antibody revealed a dynamic role of miRNA-mediated translational control in various developmental and physiological programs. Based on our data and previous findings, we proposed that phosphorylation of AGO1 is essential for the miRISC to function as a translational repressor.

Introduction

Small RNAs in plant development, hormone signaling and seed germination

In plants, miRNAs specifically base-pair to target mRNAs to either repress the target translation or induce subsequent cleavage (Bartel, 2004). A number of lines of evidence have implicated miRNAs in many aspects of plant development. First of all, mutations in genes encoding the core enzymes critical for miRNA biogenesis, such as DCL1, HYL1, HEN1, and the RISC core enzyme AGO1, result in pleiotropic defects throughout the plant life span (Jacobsen et al., 1999; Lu and Fedoroff, 2000; Park et al., 2002; Vaucheret et al., 2004). Secondly, target mRNAs of many plant miRNAs encode transcription factors that are involved in developmental patterning and cell differentiation (Rhoades et al., 2002; Kidner and Martienssen, 2005). For instance, miR319 has been shown to control the expression of TCP transcription factors that affect leaf morphologensis in several model systems (Palatnik et al., 2003; Ori et al., 2007). Another conserved miRNA family, namely MiR165/166, regulates the HD-ZIP transcripion factors to govern the correct establishment of the adaxial/abaxial leaf axis as well as vascular development in Arabidopsis and maize (Emery et al., 2003; Juarez et al., 2004; Kidner and Martienssen, 2004; Kim et al., 2005).

Even before the characterization of the components of the miRNA biogenesis pathway, several embryo-defective mutants, namely *sus1*, *sin1* and *caf*, had been described. These were later shown to be allelic to *dcl1* (Lang et al., 1994; Ray et al., 1996; Jacobsen et al., 1999; Golden et al., 2002; Schauer et al., 2002). In *dcl1-15* mutants (mutants defective in miRNA biogenesis), many positive regulators of embryo maturation, including FUS3

(FUSCA3) and LEC2 (Leafy-Cotyledon2), were up-regulated, and triple mutants of *dcl1-15/fus3/lec2* displayed an alleviated *dcl1-15* phenotype. The rescued *dcl1-15* phenotype of *dcl1-15/fus3/lec2* triple mutants indicated that the loss of repression of FUS3 and LEC2 in *dcl1-15* mutants was likely the reason for the embryo-defective phenotype observed in *dcl1-15* mutants (Willmann et al., 2011a). Moreover, plants with mutations in other components that are responsible for miRNA biogenesis, such as SE, HYL, and HEN1, also displayed various degrees of defective embryogenesis (Chen et al., 2002; Vazquez et al., 2004a; Lobbes et al., 2006; Yang et al., 2006a). Altogether these findings suggested important roles of miRNAs during embryo maturation and seed development. One pronounced example came from the study of miR164. In Arabidopsis, miR164 targets five members of the NAC (No Apical Meristem; ATF1/2 and Cup-Shaped Cotyledon2) transcription factor family (Mallory et al., 2004a; Raman et al., 2008). Arabidopsis transgenic lines overexpressing miR164 exhibited cup-shaped cotyledons (Mallory et al., 2004a). Concomitantly, in plants expressing CUC2 (Cup-shaped Cotyledon 2) with mutated miR164 target sites (miR164 would no longer be able to target these mutated CUC2 transcripts for degradation), enlarged meristem boundary domains were found in shoot apices. Therefore, results from these two reciprocal experiments demonstrated the importance of miR164-directed regulation of CUC1/ CUC2 for proper embryo development (Laufs et al., 2004).

In addition to their roles in development, plant miRNAs have also been implicated in multiple hormone signaling pathways. In Arabidopsis, miR159 regulates genes encoding proteins belonging to a GAMYB transcription family, and is essential for proper flower

timing, anther development and seed germination. During floral and anther development, the expression of miR159 is induced by gibberellic acid (GA) to down-regulate the cognate MYB33 and MYB64 transcription factor mRNAs via opposition of DELLA function (DELLA: GA-regulated factors; for review on GA and DELLA proteins, see Jiang and Fu, 2007). Conversely, in the absence of GA, the DELLA proteins are expressed and through some unclear mechanism dampen the activity of miR159 (Achard et al., 2004). Auxin signaling pathways have also been shown to be under the control of miRNAs. For instance, different auxin response factors (ARFs) are regulated by miR160 and miR167, to modulate biological processes such as gametogenesis, seed germination, and nutrient uptake (Sun; Sunkar and Zhu, 2004; Millar and Gubler, 2005; Wang et al., 2005; Wu et al., 2006; Liu et al., 2007). Another example came from studies of miR393 and its targets TIR1 (Transport Inhibitor Response1) and the TIR1 paralogs ABF2 and ABF3 (Auxin Signaling F-box2/3; Rhoades et al., 2002). In Arabidopsis, TIR1 is an F-box protein in the Skp, Cullin, F-box containing complex (SCF complex), which mediates the ubiquitination of AUX/IAA proteins and their subsequent proteolysis via the 26S proteasome pathway (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The TIR1-mediated degradation of AUX/IAA proteins releases ARFs and triggers a series of downstream auxin responses (reviewed in Reed, 2001) . Therefore, miR393-mediated down-regulation of TIR1/ABF dampens auxin responses. More recently this pathway has been implicated in antibacterial resistance, nitrate-responsive root architecture establishment, and auxin-dependent leaf development (Navarro et al., 2006; Vidal et al., 2010; Si-Ammour et al., 2011).

Plant hormones have been known to be one of the key regulators during seed development and germination. As reviewed briefly earlier in this chapter, miRNAs are interwoven in several hormonal signal transduction pathways. Generally speaking, seed dormancy/germination status is determined by the balance between GA and ABA (reviewed by Koornneef et al., 2002). As summarized in Figure 3.1, during seed germination, ABA induces the production miR159 to negatively regulate the GAMYB transcription factors MYB33, MYB65 and MYB101, which are the positive regulators of ABA responses. Therefore, this modulated pathway creates a homeostatic mechanism, which is essential for proper germination and growth control (Reyes and Chua, 2007; Alonso-Peral et al., 2010). Interestingly, in both Arabidopsis and cereals, GAMYB transcription factors have been shown to be induced by GA in aleurone cells (Gubler et al., 1999; Bethke et al., 2007; Guo and Ho, 2008). These GAMYB transcription factors not only activate the production of many hydrolases that are responsible for the mobilization of nutrients in the endosperm, but also promote the aleurone vacuolation, a PCD process in aleurone cells that is required for germination (Gubler et al., 1999; Bethke et al., 2007; Guo and Ho, 2008; Alonso-Peral et al., 2010).

The miR160/ARF10 regulatory module is also involved in the seed germinative process (Liu et al., 2007). In seeds overexpressing miR160-resistant ARF10 mRNAs (ARF10 with a mutated miR160 target site, which renders ARF10 resistant to miR160-directed cleavage), the sensitivity to ABA was drastically increased, resulting in a reduced rate of radicle emergence compared to controls under low ABA concentration (Liu et al., 2007). By contrast,

transgenic seeds overexpressing miR160 exhibited reduced ABA sensitivity (Liu et al., 2007). Interestingly, exogenous IAA reversed the reduced sensitivity to ABA in the seeds overexpressing miR160. Even though auxin was not known to be the main player during seed germination, this ARF10-confered ABA hypersensitivity suggested a potential cross-talk between ABA and auxin responses during seed germination (Liu et al., 2007).

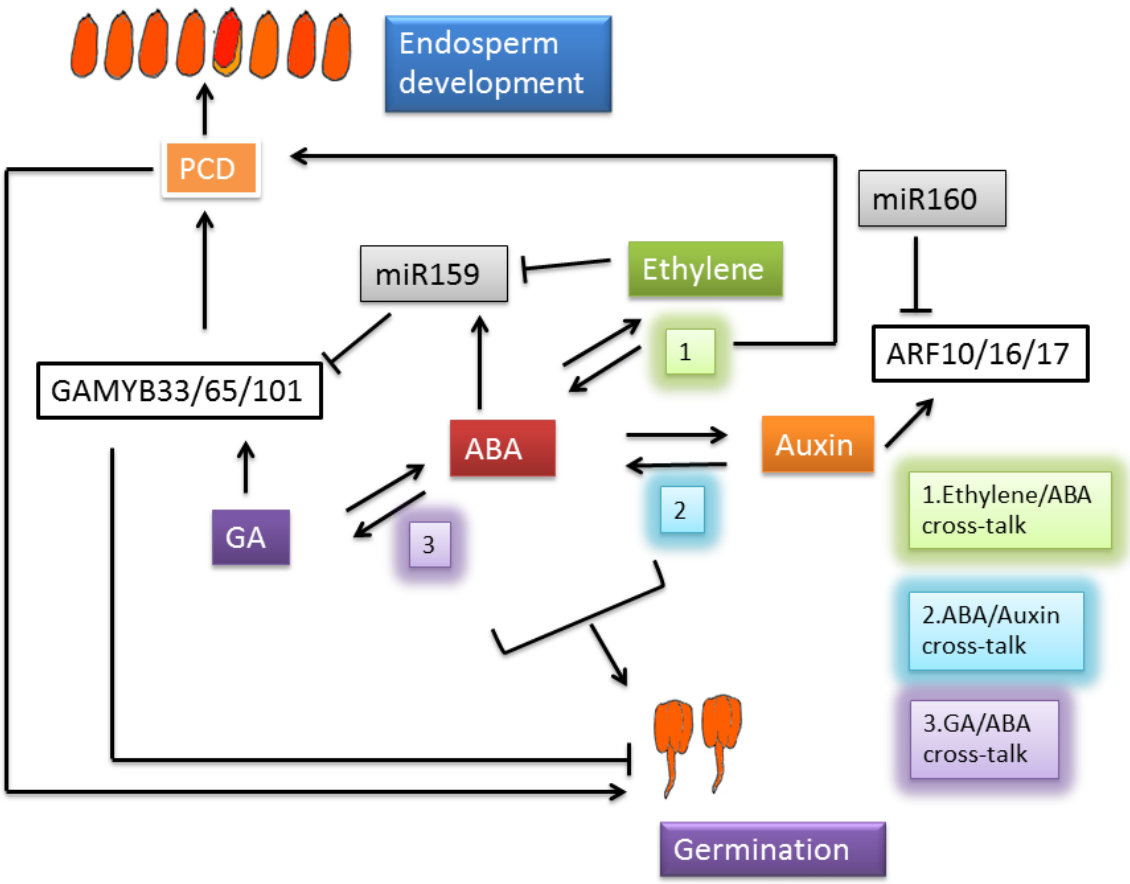


Figure 3.1 Representative miRNAs in hormonal signalling networks during seed development and germination.

MiR159 and miR169 are two of the most studied miRNAs involved in seed biology.
PCD: Programmed cell death; ABA: Abscisic acid; GA: Gibberellic acid.

Translational repression in plants and its potential role in seed development and germination

Since the initial finding that, in Arabidopsis, miR172 uses both cleavage and translational repression as regulatory mechanisms (Aukerman and Sakai, 2003; Chen, 2004; Lauter et al., 2005), it has been a matter of debate how much translational repression contributes to the

total plant miRNA activities. Unlike in animals, our mechanistic understanding of this particular miRNA-mediated mechanism in plants has just started to emerge. In Arabidopsis, in addition to the major miRNA effector AGO1, AGO10 was also proposed to be involved in translational repression by miRNAs due to the disproportionately higher protein levels compared to mRNA levels of miRNA targets in *ago10* mutants (Brodersen et al., 2008). Consistent with the finding that, in animals, microtubules were involved in the miRNA-mediated gene repression (Cook et al., 2004; Parry et al., 2007), a mutant screening in Arabidopsis and the subsequent characterization revealed a role of a microtubule-severing protein, namely KTN1, in miRNA-directed translational repression (Brodersen et al., 2008). The discovery that in animal and plant systems, microtubule dynamics were required for miRNAs to translationally repress target expression suggested that perhaps an evolutionarily conserved mechanism for miRNA-mediated translational regulation was present in these two kingdoms. In line with this idea, components in decapping complexes have also been shown to be required in both animals and plants to direct translational arrest of target mRNAs (Eulalio et al., 2007c; Brodersen et al., 2008; Motomura et al., 2012).

Back in the 1970s, Chen et al. reported the effects of GA and ABA on protein translation during early germination of wheat embryos (Chen and Osborne, 1970). They observed that while neither GA nor ABA affected the pattern of monosome and polysome distribution, the rate of protein synthesis in polysomal fractions was altered by the hormone treatments. By pulsing the wheat embryos with a 14C-amino-acid mixture, Chen et al. observed an enhanced level of aminoacyl-tRNAs associated with the ribosomes from GA-treated embryos, while

the ribosomes isolated from ABA-treated embryos manifested reduced levels of radioactivity (Chen and Osborne, 1970). Interestingly, this translational control seemed to affect only the stored mRNAs rather than the mRNAs synthesized *de novo* during embryo germination (Chen and Osborne, 1970). This discovery, together with several more recent findings, indicated the presence of a certain form of translational control during the transition from a quiescent to a metabolically active state during seed germination (Dinkova et al., 2000; Dinkova TD, 2003; Rajjou et al., 2004).

One mechanism underlying this observed translational control during seed germination could be attributed to miRNA activities. First of all, as discussed earlier in this chapter, many miRNAs have been demonstrated to be crucial for a variety of developmental programs including seed germination. Secondly, it has been reported that instead of completely silencing the target expression as observed in seedling and vegetative tissues, during Arabidopsis seed germination, miR159 affected the target transcript levels of GAMYB33/65 to only a modest level (Alonso-Peral et al., 2012). And, this attenuation of miR159 activity was independent of miR159 abundance because the level of mature miR159 remained relatively stable in the early stages of seed germination and developing seedlings. Intriguingly, while the GAMYB33 transcript levels at 5 hr, 16 hr and 24 hr after imbibition (AI) were shown by RT-qPCR to be relatively similar, in transgenic seeds expressing a GAMYB33:GUS fusion protein, the fusion protein was barely detectable at 5 hr AI but became more obvious after 16hr AI (Alonso-Peral et al., 2012). These findings suggested that

a translational control of GAMYB33, potentially imposed by the activity of miR159, was present during the early stages of seed germination.

Based on all the aforementioned experimental results and observations, we hypothesized that miRNAs are involved in regulating an array of biological processes during seed development and germination. The fact that we were able to find miRNA species that were preferentially distributed in the polysomal fractions (as discussed in Chapter 2) suggested the presence of a regulatory mechanism wherein miRNAs repress target expression co-translationally via directly interfering with the transcripts after the loading of ribosomes. Therefore, we firstly verified the deep sequencing data (as discussed in Chapter 2) by RT-qPCR. Then, based on this knowledge, we sought to profile the expression pattern of miRNAs that were detected in our deep sequencing study from both maize endosperm and embryos at different developmental and germinative stages. In order to understand mechanistically how plant miRNAs regulate protein translation, we took advantage of an Arabidopsis transgenic system expressing a FLAG-tagged ribosomal protein RPL18. By performing immunoprecipitation studies with anti-FLAG and anti-AGO1 antibodies respectively, we sought to test if the translational machinery interacted with miRISCs *in vivo*. Indeed, we found that not only was the antibody against FLAG-tagged RPL18 able to pull down the AGO1 protein, but also many miRNAs were associated with the ribosome-AGO1 complex. Therefore we were able to demonstrate the co-localization of the functional miRISC and actively translating polysomes in the same protein complex. Moreover, immunoprecipitation experiments with anti-AGO1 revealed that in dry Arabidopsis seeds, miRNA association with ribosomes was

minimal, but once germination started, more miRNAs were found to be interacting with ribosomes. In 20-day old seedlings, we showed that miRNAs pulled down by AGO1 antibodies from ribosomal fractions were even more abundant than those from non-ribosomal fractions. All of these findings suggested a dynamic role of miRNA-mediated translational control in various developmental and physiological programs.

In the discussion section, based on our data and previous findings, we propose a potential mechanism by which miRISCs translationally repress target protein production. Within this model, we suggest that phosphorylation of AGO1 is essential for the miRISC to function as a translational repressor. Subsequent computational analyses of AGO1 phosphorylation revealed a localized phosphorylated serine in the PIWI domain of AGO1. This serine residue seemed to be conserved among AGO1 proteins from different species, and it frequently underwent phosphorylation. Interestingly, it was located close to the histidine residue in the "DDH" catalytic motif, which is responsible for the slicing activity of AGO1 proteins. Based on all the collective evidence, we propose that the phosphorylation of AGO1 on this serine residue inhibited the slicing activity by interfering with the catalytic histidine in the "DDH" motif, thus enabling the miRISC to act as a translational repressor without apparent slicing effects on its targets.

Results

RT-qPCR verification of Next-Gen sequencing results

In Chapter 2, we discussed our experimental results obtained from a Next-Gen sequencing study. By sequencing small RNAs isolated from ribosome-associated fractions and ribosome-free fractions, we sought to identify miRNAs that were preferentially associated with the translational machinery in 18 DAP maize endosperm. We hypothesized that these ribosome-associated miRNAs potentially acted as translational repressors to regulate gene expression by directly interfering with target protein synthesis. In this chapter, we firstly aimed to experimentally verify our discoveries from the Next-Gen sequencing study.

To verify the differential distribution of various miRNAs detected in our Next-Gen sequencing results, we employed miRNA- specific stem-loop PCR (Chen et al., 2005). In short, ribosomal fractions were isolated from the non-ribosomal fractions by differential ultracentrifugation. Ribosomal-associated RNAs were collected in the pellet and ribosome-free RNAs in the supernatant. To confirm the validity of this ribosome isolation method, a western blot was performed using proteins extracted from the crude homogenate (total protein before the separation), the supernatant and the ribosomal pellet. An antibody against the maize ribosomal subunit protein S6 was used to probe for the presence of ribosomes. As shown in Figure 3.2, S6 protein was present in both the crude homogenate and ribosomal fractions but not in the supernatant. Thus, the ultracentrifugation was effective in separating the ribosome-associated RNAs from ribosome-free fractions.

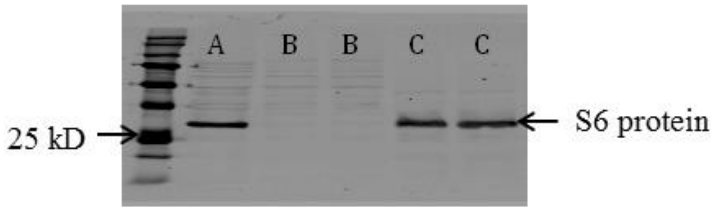


Figure 3.2 Western blot showing the depletion of the ribosomal protein S6 from non-ribosmoal fractions.

Ribosome-associated fractions from approximately 2 grams of 18DAP endosperm were isolated from ribosome-free fractions by ultracentrifugation. Total proteins extracted from the crude homogenate, ribosomal and non-ribosomal fractions were separated by SDS-PAGE (15%) prior to transfer and probing with antibodies against the maize ribosomal protein S6.

A.Crude homogenate; B and C. Non-ribosomal and ribosomal fractions from two biological replicates, respectively.

The arrow on the left points at the molecular weight marker of 25kD; The arrow on the right indicates the band of the S6 protein, which is approximately 27kD.

Figure 3.3 shows the comparison of the most abundant miRNAs from Next-Gen sequencing (Chapter 2) and the same miRNAs quantified by miRNA RT-qPCR assay. Consistently, results of the miRNA RT-qPCR assay reproduced previous findings, with the exception of the ribosomal level of miR167. In ribosomal fractions, our sequencing data suggested that miR168 was nearly twice as abundant as miR166 and miR319, and the miRNA RT-qPCR results verified that indeed the level of miR168 exceeded that of miR319 and miR166. Surprisingly, expression data for miR167 from the sequencing and qPCR assay did not align as expected, with the qPCR result exhibiting a much higher abundance than the sequencing data. This discrepancy might result from a shadowing effect that occurred during the

sequencing of one of the ribosomal replicates. As discussed in Chapter 2, we observed an over-representation of rRNAs in the ribosomal fraction from biological replicate 1. Consequently, the detection of many miRNAs was obscured by the presence of a large amount of rRNA. As for the non-ribosomal fractions, results of miRNA abundance obtained by RT-qPCR in these fractions recapitulated that of our sequencing study, with miR167 being the most abundant miRNA, followed by miR168 and miR166.

Interestingly, both our sequencing and qPCR data revealed that miR319 had a disproportionally high distribution in ribosomal fractions. As shown in Figure 3.3, while the other miRNAs displayed ribosomal abundance that was proportional to their non-ribosomal abundance, miR319 exhibited a preferential distribution in ribosomal fractions. Therefore, we postulated that this preferential distribution of miR319 might reflect its robust function in interacting with the translational machinery to modulate the translation of cognate mRNAs.

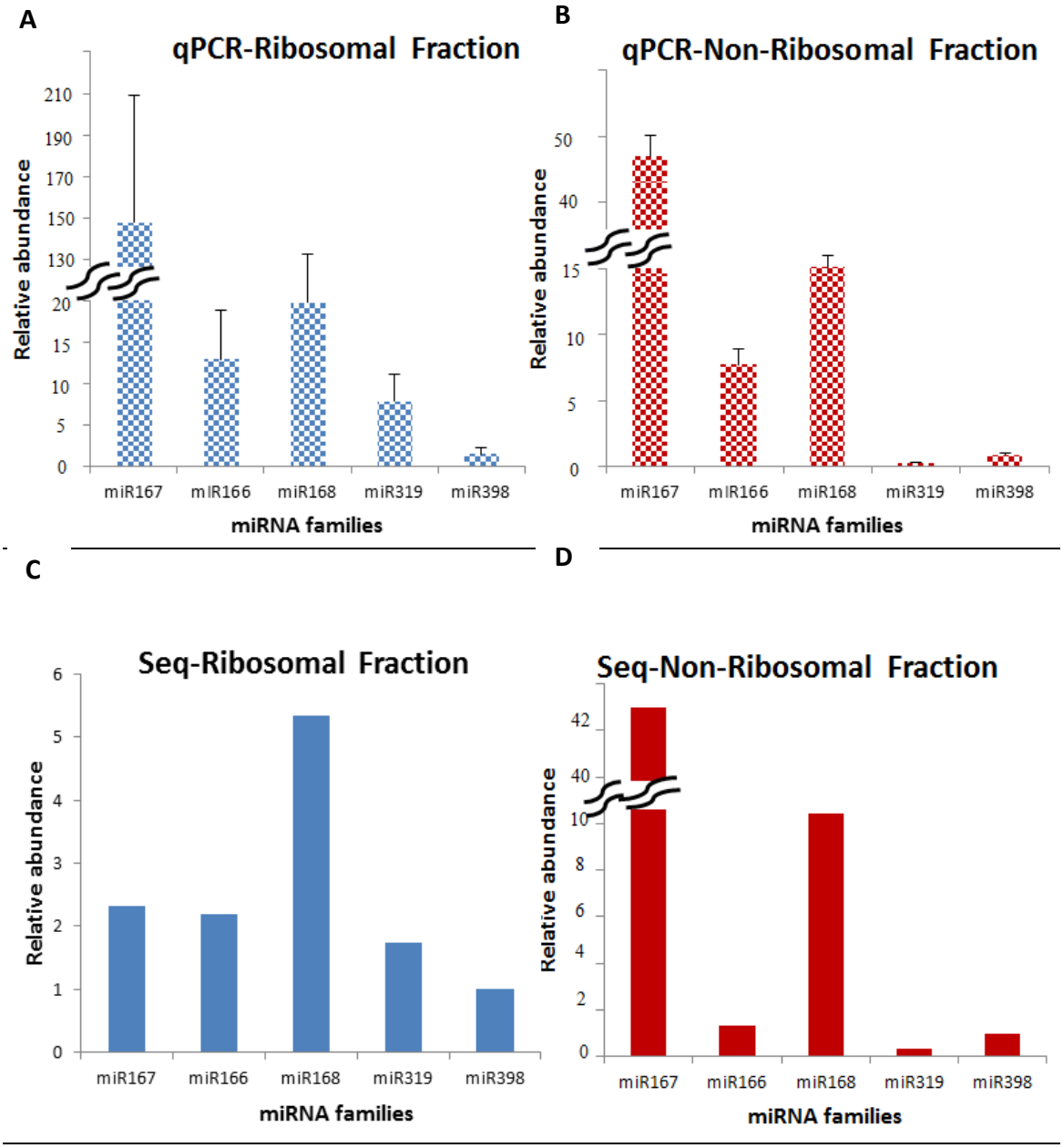


Figure 3.3 Comparison of ribosomal and non-ribosomal miRNA abundance as measured by Next-Gen sequencing and miRNA RT-qPCR assays.

A and B. The relative abundance of selected miRNA families detected by miRNA RT-qPCR from ribosomal and non- ribosomal fractions, respectively. C.and D. The relative abundance of selected miRNA families detected by Next-Gen sequencing from ribosomal and non-ribosomal fractions, respectively.
All of the miRNA levels were normalized by the level of miR398 in each sample.

Translational repression by miRNAs can occur after translation initiation

To gain better resolution of the distribution of miRNAs across polysomes, we fractionated the ribosome-associated RNAs on sucrose gradients by differential ultracentrifugation. Polysome profiles (such as those in Figure 3.5 A, see supplemental data for all the profiles) were generated by fractionating the sucrose gradient through a UV detector. Individual fractions were collected and pooled to generate five to six polysomal samples. RNAs extracted from these fractions were used for the miRNA RT-qPCR assay. Figure 3.4 shows the results obtained from these RT-qPCR studies. Here polysomes were fractionated and pooled into five fractions (F1 through F5), with F1 containing the two ribosomal subunits and monosomes, and F3 and F4 containing the large polysomes (as shown in Figure 3.5 A). As shown in Figure 3.4, despite the fact that different miRNAs exhibited various degrees of ribosomal association, they were all found in fractions containing large polysomes such as F3, F4 and F5. This observation was particularly interesting in light of our hypothesis that in developing maize endosperm, miRNA-mediated translational control occurred after the translation initiation step. We favored this hypothesis over other models for the two reasons. First of all, levels of miRNAs were much lower in the F1 fractions, which contained only ribosomal subunits and monomers (Figure 3.4). Low abundance of miRNAs in these fractions suggested that either the association of miRISCs with ribosomal subunits and monomer ribosomes was transient, or this interaction did not predominantly occur during 80S ribosome assembly. Secondly, the majority of miRNAs was found in large polysomes. Should the miRNA-directed regulation take place during the process of translational

initiation, the target mRNAs would not enter the large polysomes. Consequently, only the minimal amount of miRNAs, if any, should be found to co-localize in large polysomes.

It is worth noting here that a higher portion of the miR319 population was found across all polysomal fractions. Unlike in the cases of miR167, miR166 and miR398, where levels of non-polysomal miRNAs were at least 30-fold higher than those in polysomal fractions, the miR319 abundance in polysomal fractions accounted for as much as 40% of that in the non-polysomal fraction. As proposed in our hypothesis, a higher degree of ribosomal association suggested stronger translational regulation imposed by this miRNA. Therefore, our finding that miR319 displayed a favored distribution across polysomal fractions might, indeed, hint at its robust role in repressing the translation of its mRNA targets, possibly via interfering with the translational elongation/ribosome translocation processes.

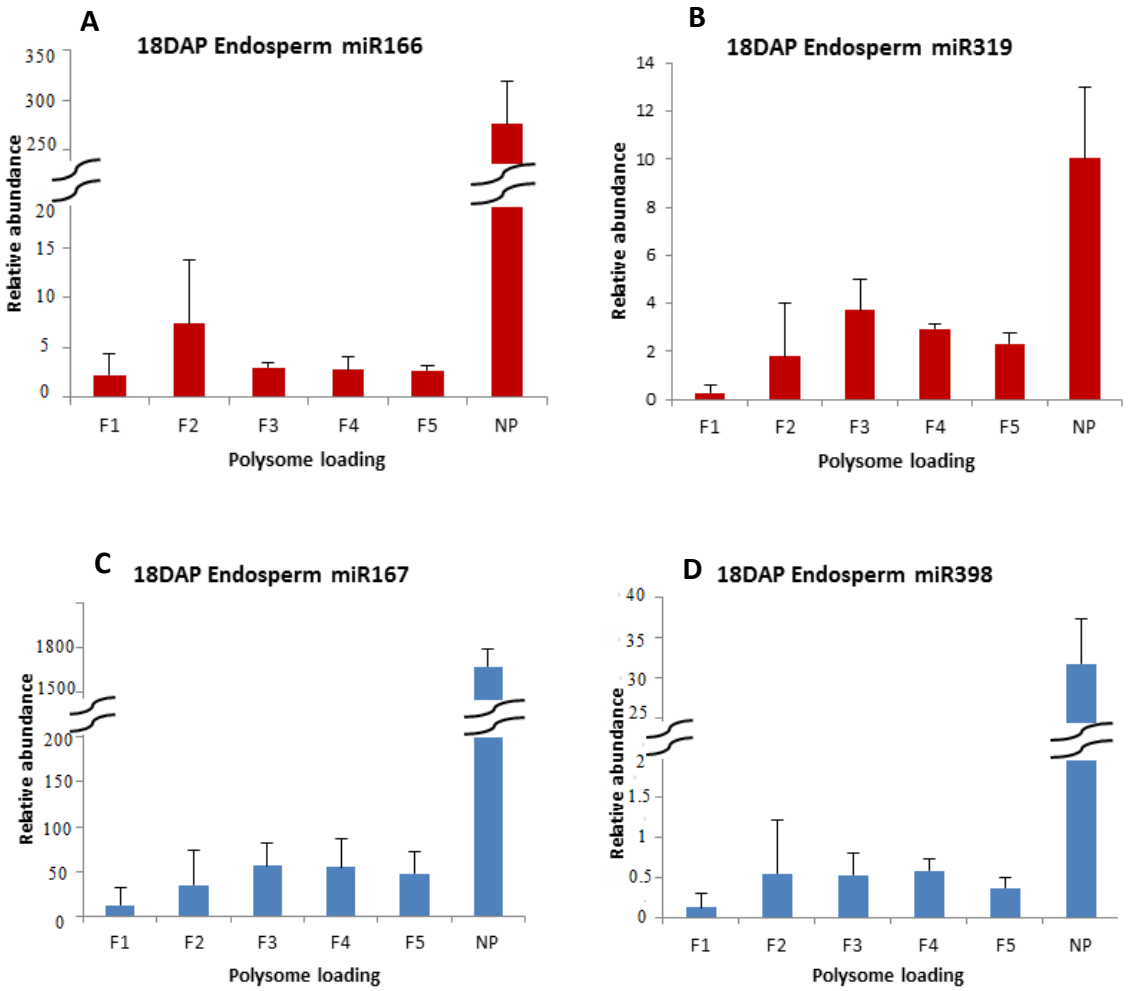


Figure 3.4 Relative abundance of various miRNAs in fractionated polysomes and non-polysomal fractions from 18 DAP endosperm.

F1-F5: Fractionated polysomes with increasing sizes. NP: Non-polysomal fraction. A. MiR166 levels in polysomal and non-polysomal fractions. B. MiR319 levels in polysomal and non-polysomal fractions. C. miR167 levels in polysomal and non-polysomal fractions D. MiR398 levels in polysomal and non-polysomal fractions. MiRNAs in red indicate they were preferentially found in ribosomal fractions from our deep sequencing study. MiRNA levels in each fraction were normalized to exogenously spiked miRNA hsa-let7.

To verify that the co-sedimentation of miRNAs with polysomes was not due to experimental artifacts that occurred during ultracentrifugation, we performed puromycin treatments on the ribosome-enriched fractions before subjecting them to sucrose density ultracentrifugation. Puromycin is an antibiotic that structurally resembles the 3' end of aminoacylated tRNAs and competes with functional aminoacylated tRNAs, resulting in the premature termination of nascent peptide chain formation (Pestka, 1971). It has been widely used as a translation inhibitor, and the application of puromycin dissociates actively translating polysomes into ribosomal subunits and monomers. As shown in Figure 3.5, we found that even though the puromycin treatment resulted in only a partial dissociation of polysomes, after the treatment, the distribution of miR167 across polysomal fractions shifted to the fractions with smaller polysomes. Release of miRNAs from polysomes by puromycin confirmed our previous conclusion that miRNAs potentially interfered with targets that were being actively translated in the polysomes.

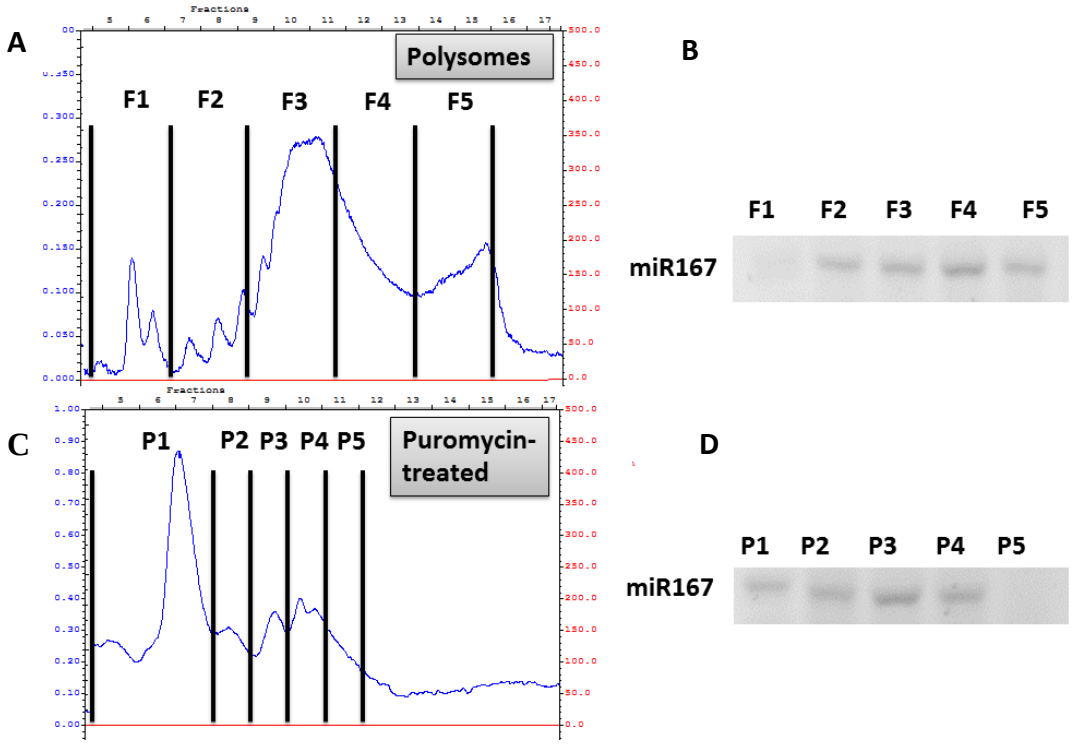


Figure 3.5 Northern blot of miR167 across different polysomal fractions.

Polysomes were fractionated through sucrose gradients by differential ultracentifugation. RNAs were recovered using Trizol-LS. F1- F5/P1-P5: Fractionated polysomes with increasing sizes. A. The profile of polysomes generated by sucrose density ultracentrifugation. B. Northern blot showing miR167 distribution across polysomal fractions. C. The polysome profile after puromycin treatment. D. Northern blot showing miR167 distribution after puromycin treatment. Pooled fractions are indicated by bars are shown in panels A and C.

Comparative distribution of miRNAs in embryos and endosperm

After establishing the expression patterns of many miRNAs from 18 DAP endosperm via deep sequencing and RT-qPCR assays, we proceeded to test if the patterns held true for the corresponding embryo tissues. Polysomal and non-polysomal RNAs from 18 DAP maize embryos were isolated and used for the RT-qPCR assay. The results are summarized in

Figure 3.6. We noticed that the relative abundance of the tested miRNAs was different in 18 DAP endosperm and embryos. For example, the expression levels of miR167 in both polysomal and non-polysomal fractions from embryos were approximately 1/3 of those in the endosperm (Figure 3.4 A and Figure 3.6 A). In contrast, the miR166 level in embryos was nearly four times higher than it was in endosperm, as reflected in both the polysomal and non-polysomal fractions (Figure 3.4 C and Figure 3.6 C).

The expression levels of miR319 also differed in these two tissues. As shown in Figure 3.4 B and Figure 3.6 B, the overall abundance of miR319 in embryos was approximately 20 times higher than it was in endosperm. However, even though the expression levels varied, the distribution pattern of miR319 across polysomal and non-polysomal fractions persisted. In both embryos and endosperm, miR319 exhibited higher polysomal distribution than any of the other miRNA tested.

These results lead us to suggest that, first of all, in endosperm and embryos, the demands for the activity of each miRNA might echo the physiological needs and biological processes in these tissues. For instance, the expression level of miR166 in embryos was higher than it was in endosperm. This difference suggested that miR166 was more robustly involved in embryogenesis at the developmental stage of 18 DAP. In agreement with this notion, it has been reported that the miR166 targets, rld1 and rld2, were involved in the development of plant shoot apical meristem, and the establishment of abaxial/adaxial polarity during embryogenesis (Williams et al., 2005; Byrne, 2006). By contrast, the relatively higher level

of miR167 in endosperm tissue suggested that auxin signal transduction was crucial for proper endosperm development, as it was shown that miR167 mediated the expression of several ARF genes in maize (Xing et al., 2011). In fact, it has been long known that auxin is responsible for inducing events such as endoreduplication and expression of particular zein storage proteins in maize endosperm (Lur and Setter, 1993). Secondly, the observation of a much stronger polysomal association for miR319 than any other miRNA tested in this assay might suggest that miR319 had a unique role in repressing the translation of its targets. In maize, miR319 was predicted to target genes encoding several proteins including a MYB-like protein (GRMZM2G028054_T01), a TCP transcription factor (GRMZM2G089361_T01), and a ubiquitin carboxyl-terminal hydrolase (GRMZM2G388987_T01).

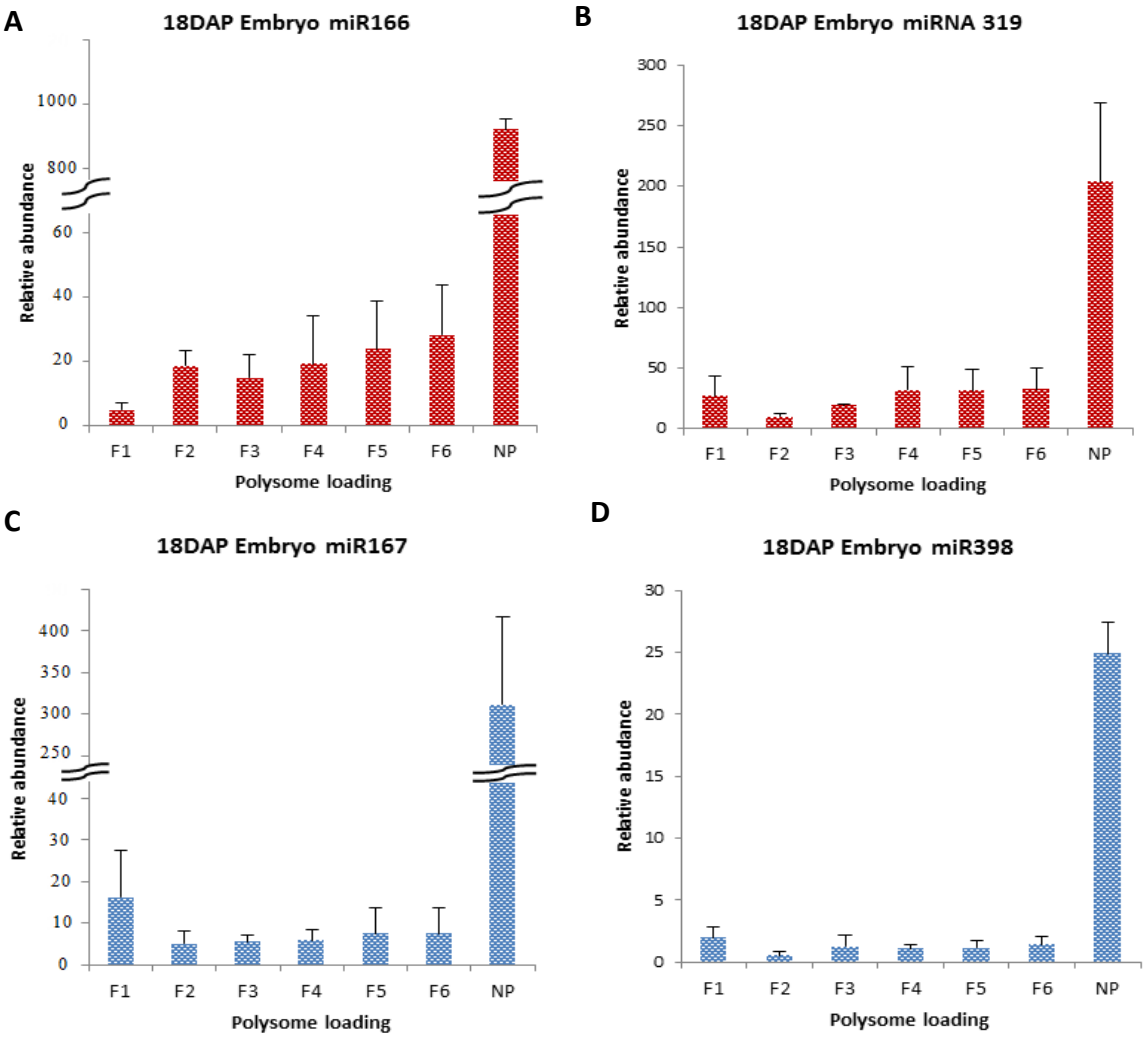


Figure 3.6 Relative abundance of various miRNAs in polysomal and non-polysomal fractions from 18 DAP embryo.

F1-F5: Fractionated polysomes with increasing sizes. NP: Non-polysomal fraction.
A. miR166; B miR319; C. miR167; D.miR398

MiRNA levels in each fraction were normalized to exogenously spiked miRNA hsa-let7.

Comparative distributions of miRNAs at different developmental stages

To investigate if miRNA abundance and the corresponding polysomal distributions also differed at different developmental stages, we compared the expression patterns of several miRNAs in both endosperm and embryos at various developmental stages (Figure 3.4, Figure 3.6 and Figure 3.7). As shown in Figure 3.7, in 10 DAP endosperm, the expression levels of miR167 and miR319 were lower than what were observed in 18 DAP endosperm (Figure 3.4 C and Figure 3.4 B). In 10 DAP endosperm, many miRNAs that later became highly expressed were maintained at a lower, apparently basal expression level (data not shown). This is very interesting because by 10 DAP, cell division has reached its peak and mitosis in endosperm becomes limited to the region directly beneath the aleurone layer (Kiesselbach, 1949; Lending and Larkins, 1989). In the meantime, the maize storage protein zein has just started to rapidly accumulate in endosperm tissues (Lending and Larkins, 1989). Therefore, the relatively low abundance of many miRNAs at this stage might be suggestive of the fact that miRNA-mediated regulation of gene expression was more dynamically involved in controlling metabolic and physiological adaptations rather than cell divisions or other events. Less difference was observed between the expression levels of miR319 and miR166 from 18 DAP and 27 DAP embryos, respectively (Figure 3.6 A, Figure 3.6 B, Figure 3.8 A and Figure 3.8 B). Moreover, when comparing the polysomal distributions of these miRNAs, we noticed that miR319 continued to exhibit a disproportionally high level of polysomal association regardless of tissue or developmental stage.

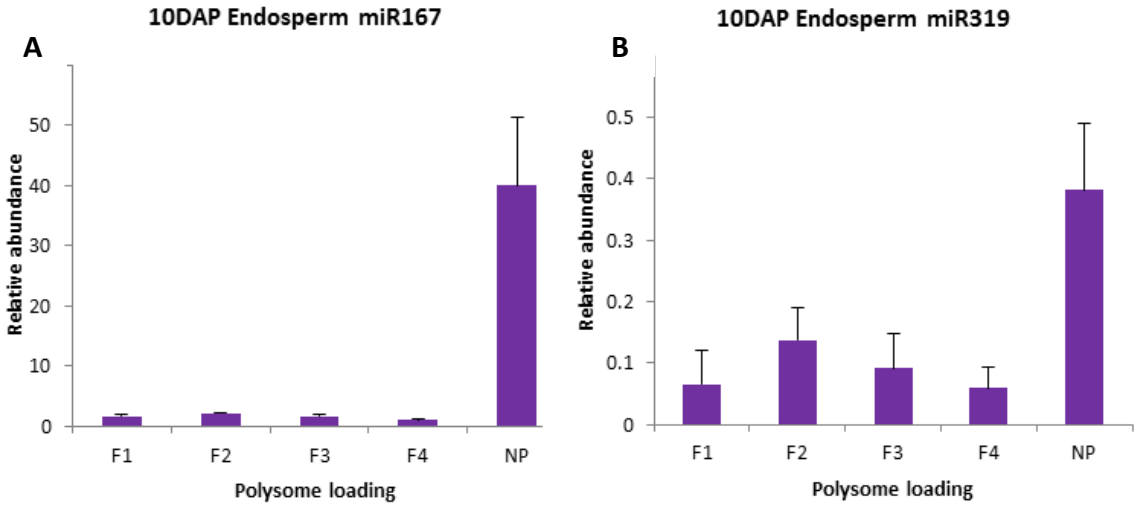


Figure 3.7 Relative abundance of miR167 and miR319 in fractionated polysomal and non-polysomal fractions from 10 DAP maize endosperm.

F1-F4: Fractionated polysomes with increasing sizes. NP: Non-polysomal fraction. MiRNA levels in each fraction were normalized by the exogenously spiked miRNA hsa-let7.

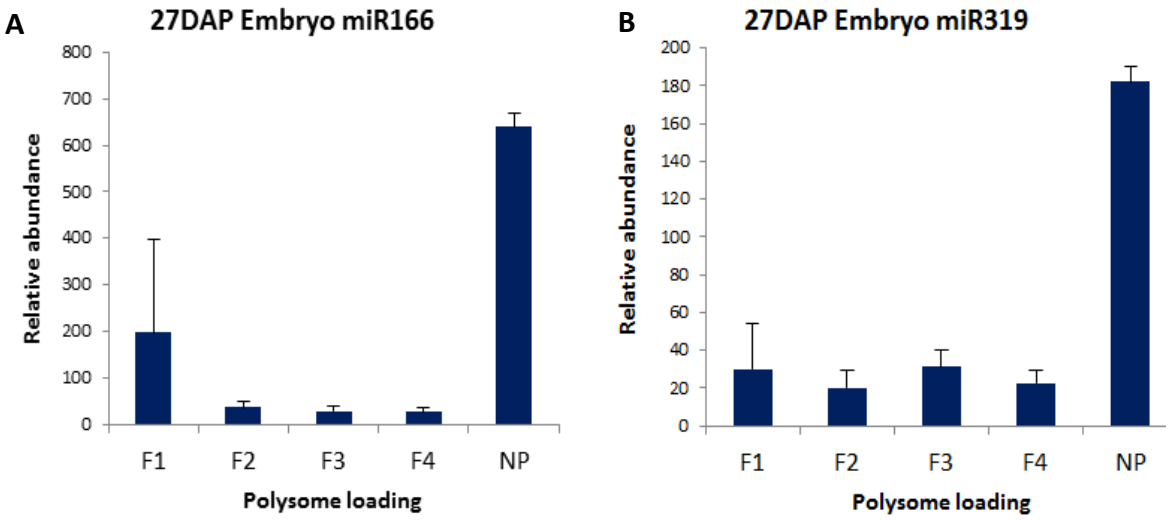


Figure 3.8 Relative abundance of miR166 and miR319 in fractionated polysomal and non-polysomal fractions from 27 DAP maize embryos.

F1-F4: Fractionated polysomes with increasing sizes. NP: Non-polysomal fraction. MiRNA levels in each fraction were normalized by the exogenously spiked miRNA hsa-let7.

MiRNAs and their targets co-localize in polysomal fractions

If miRNAs detected in the polysomal fractions were indeed interfering with the target translation, we should be able to detect the cognate miRNA targets in polysomes. To test this notion, we used RT-qPCR to quantify transcript levels of selected miRNA targets. As mentioned earlier in this chapter, miR166 has two experimentally verified targets in maize, namely Rld1 and Rld2 (Juarez et al., 2004). In addition, we tested two computationally predicted targets of miR167 in maize. As expected, we verified the presence of the miR166 targets Rld1 and Rld2, as well as the predicted miR167 ARF8-like targets in both polysomal and non-polysomal fractions from 18DAP endosperm. Collectively, these data provided

further evidence to support our hypothesis and suggested miRNAs indeed may exert their functions at the level of translation to modulate target protein production.

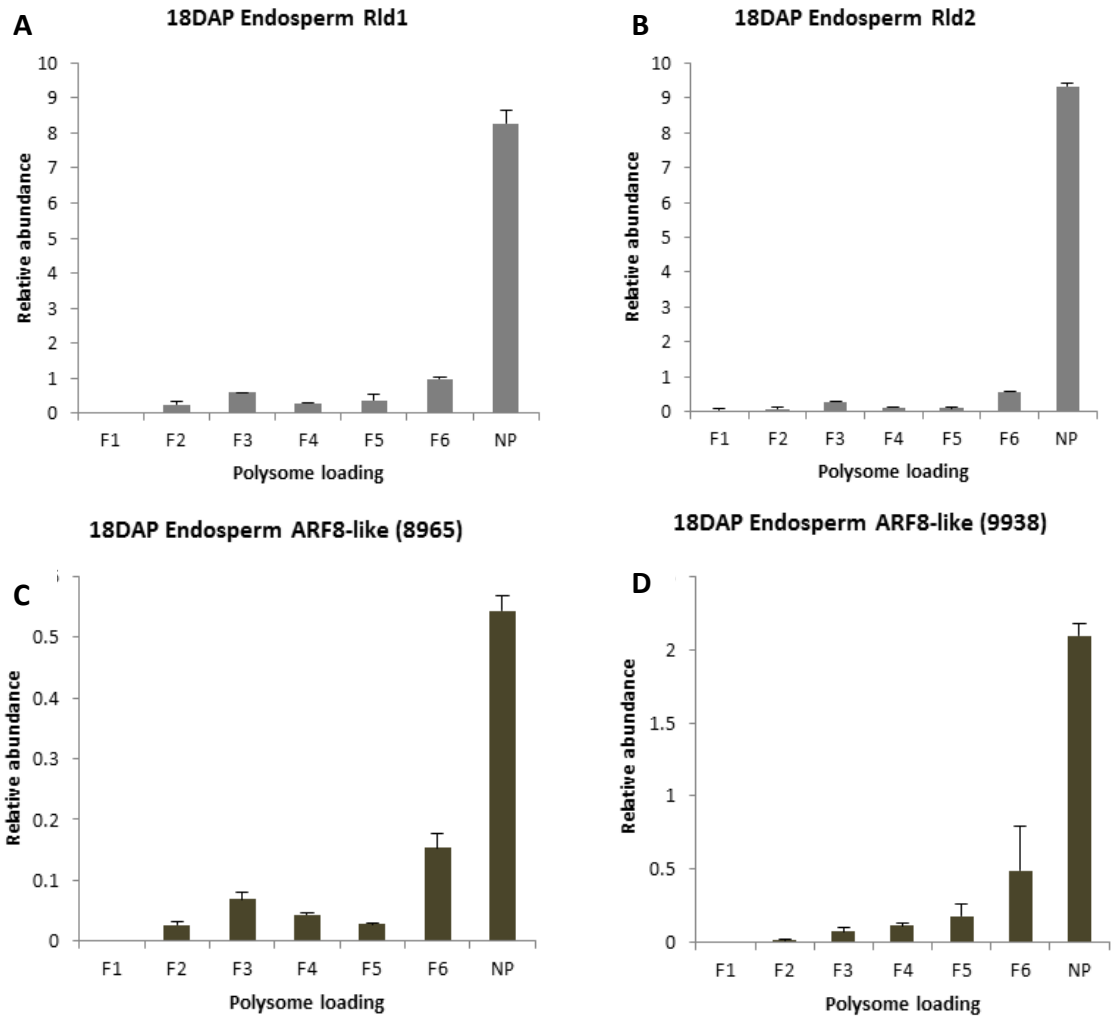


Figure 3.9 Relative abundance of miR166 and miR167 targets in fractionated polysomal and non-polysomal fractions from 18 DAP endosperm, as measured by RT-qPCR.

F1-F5: Fractionated polysomes with increasing sizes. NP: Non-polysomal fraction
Rld1 and Rld2: previously reported targets of miR166 in maize;
ARF8-like (8965) and (9938): computationally predicted targets of miR167 in maize.
Expression levels were normalized by the level of maize actin8 in the corresponding fractions.

Profiling of miRNAs during maize seed germination

Because plant miRNA activities have been suggested to be involved in regulation of proper seed germination (Liu et al., 2007; Alonso-Peral et al., 2010; Martin et al., 2010; Wang et al., 2011a; Alonso-Peral et al., 2012; Ding et al., 2012; Li et al., 2013), we sought to profile maize miRNA expression during the course of germination. Embryos and endosperm were harvested after 24 hr imbibition and at the stage when radicle protrusion was observed (2-4 days), respectively, and used to prepare ribosomal and non-ribosomal fractions by ultracentrifugation. As shown in Figure 3.10 and Figure 3.11, many miRNAs were present in ribosomal and non-ribosomal fractions from both embryos and endosperm. In embryos, the overall miRNA distribution patterns in ribosomal and non-ribosomal fractions were similar between samples harvested at the two time points. It is worth noting here, however, that miR390 displayed a unique change in distribution pattern between ribosomal and non-ribosomal fractions. At the early stage of imbibition (24hr after imbibition), miR390 was absent in the ribosomal fraction, while its level was readily detectable in the non-ribosomal fraction. However, when seeds approached the end of germination with radicles emerging from embryos, miR390 was found to be associated with ribosomes. This finding suggested that either miR390 acted in different modes, or had different sets of targets at these two stages of germination. Interestingly, in Arabidopsis and maize, miR390 is known to target the non-protein-coding transcript *TAS3*, triggering the production of trans-acting siRNAs (tasiRNAs). These tasiRNAs would in turn silence the expression of several ARFs (Montgomery et al., 2008a; Felippes and Weigel, 2009; Nogueira et al., 2009; Douglas et al., 2010; Marin et al., 2010). The fact that in our study miR390 was found to be associated with

ribosomes suggested that in maize, miR390, in addition to directing *TAS3*-dependent tasiRNA biogenesis, might have a different set of protein-coding targets.

Unlike in embryos, where no apparent difference in miRNA distribution patterns was observed between early and near-end stages of germination, in endosperm, we found that for many miRNAs, their distributions exhibited a ribosomal to non-ribosomal shift. As shown in Figure 3.11, miR156, miR159, miR169, and miR319 were present in the ribosomal fraction from the 24 hr-imbibed endosperm. However, once germination reached the end with radicles protruding from embryos, in the endosperm, the majority of these miRNAs shifted to the non-ribosomal fraction. The most extreme case was miR319. In the 24 hr-imbibed endosperm, it predominantly was found in ribosomal fractions. At the end of germination, however, the majority of miR319 was detected in non-ribosomal fractions. This finding, again, was in line with the notion that miRNAs could change their mode of action in different biological contexts. However, the mechanistic determinants that are responsible for switching the miRNA mode of operation are currently unclear.

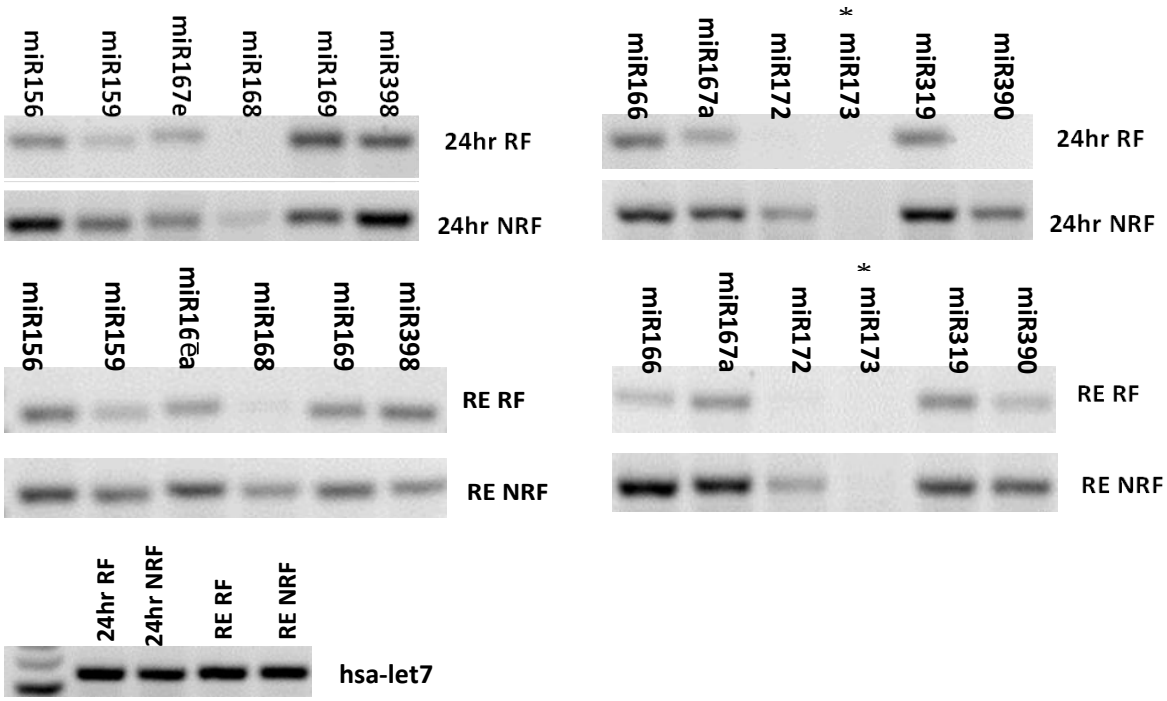


Figure 3.10 Semi-quantitative RT-PCR of miRNAs from maize embryos after 24-hr imbibition and with protruding radicles.

The ribosomal and non-ribosomal fractions were spiked with 0.1ng hsa-let7 sRNA prior to RNA extraction.

RF: Ribosomal fraction; NRF: Non-ribosomal fraction; 24hr: RNA sample extracted from embryos after 24hr imbibition in water; RE: RNA sample extracted from embryos with emerging radicles.

Because no miR173 homologous gene is present in the maize genome, Ath-miR173 was used here as the negative control for random amplification (marked by *).

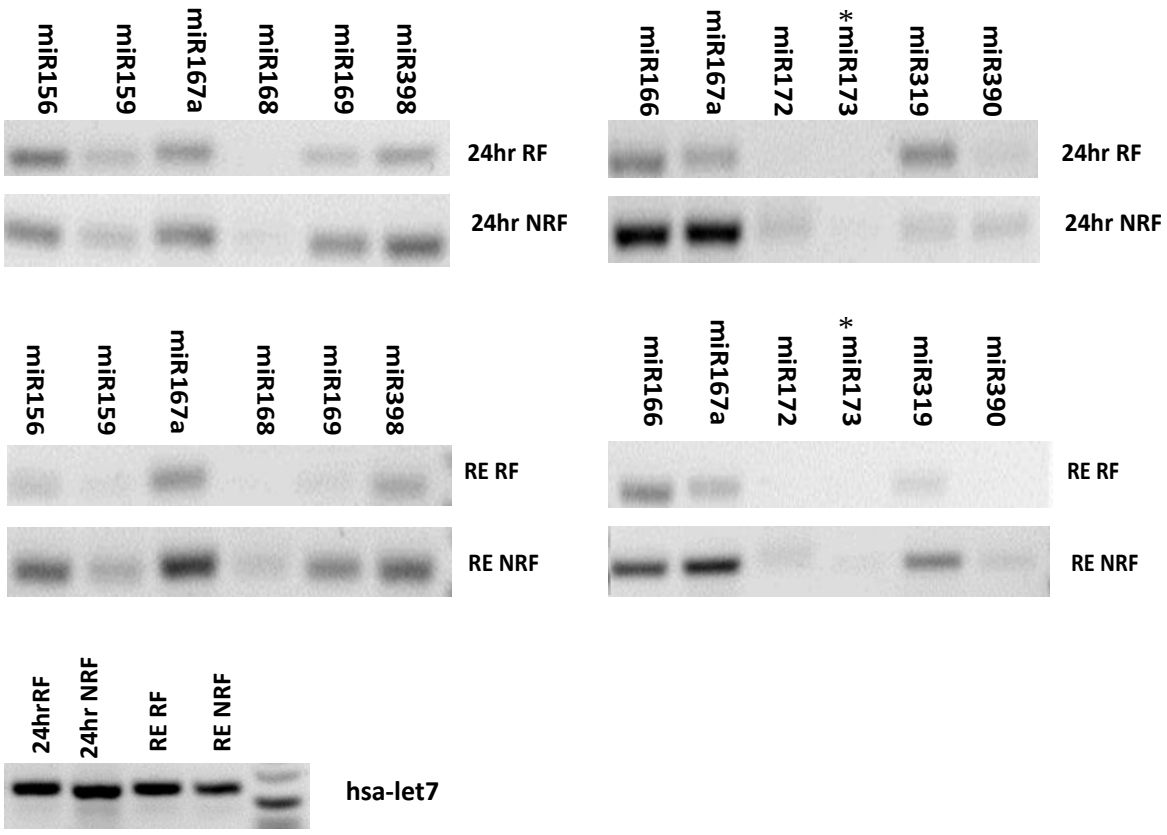


Figure 3.11 Semi-quantitative RT-PCR of miRNAs from maize endosperm after 24-hr imbibition and endosperms at the stage when radicles protruded from the embryos.

RNA samples were spiked with hsa-let7 miRNA as described in Figure 3.10.
RF: Ribosomal fraction; NRF: Non-ribosomal fraction; 24hr: RNA sample extracted from embryos after 24hr imbibition in water; RE: RNA sample extracted from embryos with emerging radicles.

Because no miR173 homologous gene is present in the maize genome, Ath-miR173 was used here as the negative control for random amplification (marked by *).

AGO1 interacts with functional ribosomes *in vivo* during Arabidopsis seed germination

To test whether or not our findings from in the maize system also extended to Arabidopsis, we proceeded to use an Arabidopsis transgenic line where a FLAG-tagged ribosome 60S subunit protein RPL18 was expressed **(**Zanetti et al., 2005**).** In this *35S-HF-RPL18* transgenic system, the anti-FLAG antibody had been shown effective in recovering polysomes by immunoprecipitation (Zanetti et al., 2005). We imbibed the *35S-HF-RPL18* transgenic seeds for 24 hours before subjecting them to immunoprecipitation by the using anti-FLAG antibody. We chose to test the same miRNAs that were assayed in our maize germination experiments in this immunoprecipitation study. As shown in Figure 3.12, many miRNAs were detected by RT-PCR from the anti-FLAG-immunoprecipitates. Furthermore, when probing the anti-FLAG immunoprecipitates with an anti-AGO1 antibody, we found that AGO1 was present only in the immunoprecipitates from the transgenic line but not in the wild-type control. To verify that the absence of miRNAs in wild-type Arabidopsis (the negative control in this experiment) was not due to compromised RNA integrity in this sample, RT-PCR was performed using the total RNA extracted from the wild-type and transgenic samples prior to immunoprecipitation. As shown in Figure 3.12 C, Arabidopsis 18S rRNA was successfully amplified in both samples, confirming that the detection of miRNAs in the anti-FLAG immunoprecipitates was indeed an outcome of miRISC-ribosome interaction.

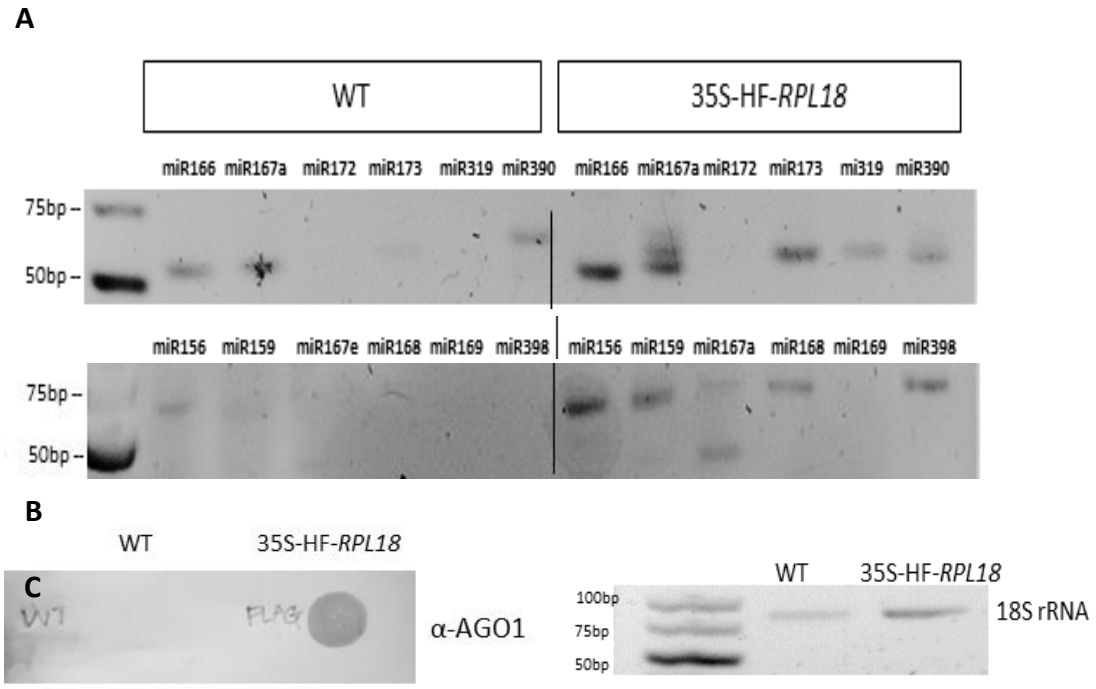


Figure 3.12 Immunoprecipitations using anti-FLAG immobilized beads and Arabidopsis seeds after 24-hr imbibition.

A: A selection of miRNAs associated with FLAG-IPs was amplified by RT-PCR. Col-0 was used as the wild-type negative control. The left lane shows the molecular weight makers of 50bp and 75bp. B: Immuno-dot-blotting using the total protein extracted from FLAG-IPs. An antibody against ath-AGO1 was used as the probe. C. 18S rRNA RT-PCR amplified from total RNA extracted from wild-type and transgenic seed prior to immunoprecipitation. The left lane shows the molecular weight markers of 100bp, 75bp and 50bp.

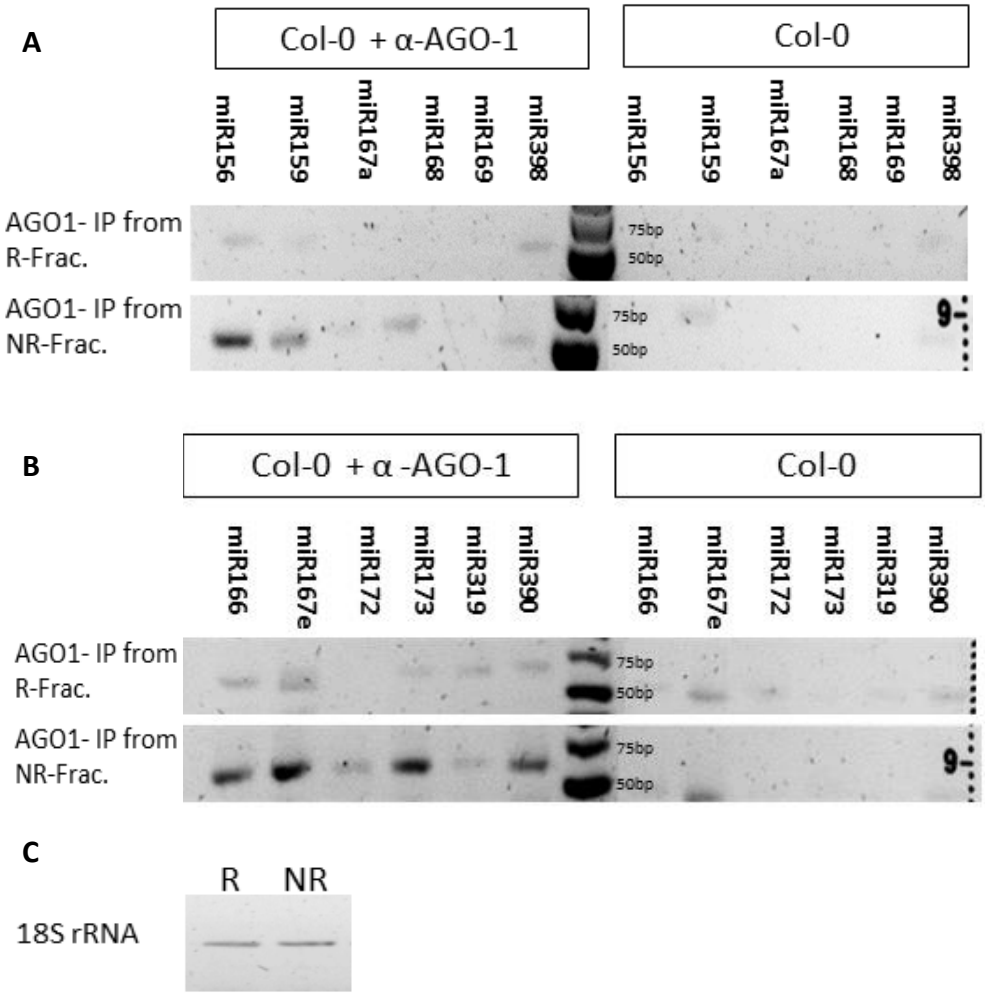


Figure 3.13 PCR amplification of miRNAs associated with anti-AGO1 immunoprecipitates in dry Arabidopsis seeds.

Dry Arabidopsis seeds were ground in extraction buffer. The ribosomal fractions were isolated from the non-ribosomal fractions by ultracentrifugation. A. miRNA RT-PCR results from AGO1-immunoprecipitates and the mock IP without adding anti-AGO1 antibodies. B. RT-PCR using the total RNA recovered from anti-AGO1-IP from ribosomal and non-ribosomal fractions. C. 18S rRNA was amplified by RT-PCR using RNAs extracted from both ribosomal and non-ribosomal anti-AGO1immunoprecipitates.
R: Ribosomsal Fraction NR: Non-Ribosomal Fraction

The size of the miRNA RT-PCR products is around 75bp. Therefore, the bands that are shown in the negative control (Col-0) are products from non-specific PCR amplification.

Dry seeds are believed to be at a state where almost all biological processes and programs are kept to a minimum. To examine how miRNAs behave in relation to their association with ribosomes under such a condition, we ground dry Arabidopsis seeds in liquid nitrogen and isolated the ribosome pellet from the ribosome-free fraction by ultracentrifugation. Then we used an anti-AGO1 antibody to perform immunoprecipitations from both ribosomal and non-ribosomal fractions. As shown in Figures 3.13 A and B, most of the miRNAs tested were present predominantly in the non-ribosomal fractions. Interestingly, Arabidopsis18S rRNA (ath-18S) was present in the AGO1-immunoprecipiates from both ribosomal and non-ribosomal fractions (Figure 3.13 C). Whether this finding indicated that ath-18S was a target of miRNA-mediated regulation or not is not clear at this moment.

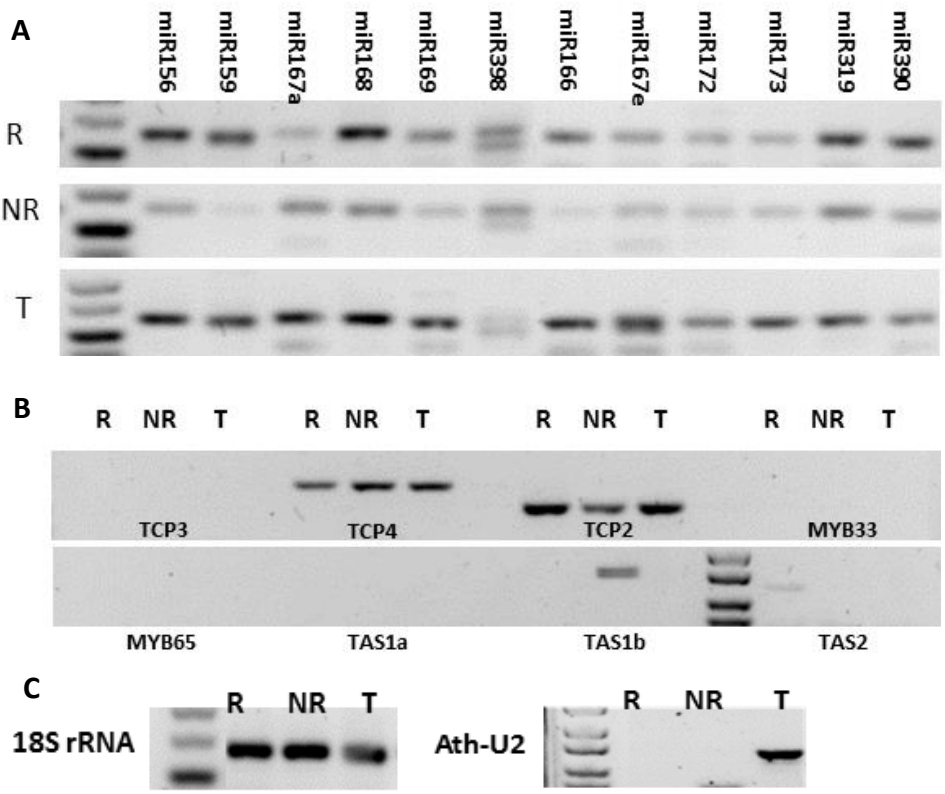


Figure 3.14 PCR amplification of miRNAs associated with anti-AGO1 immunoprecipitates in 20-day Arabidopsis seedlings.

20-day seedlings were ground in extraction buffer, and the ribosomal fraction isolated from the non-ribosomal fraction by ultracentrifugation. Anti-AGO1 was used for IP from both of these two fractions. A: miRNAs that were associated with anti-AGO1 immunoprecipitates were amplified by RT-PCR. B: miRNA targets in the AGO1-IP from both ribosomal and non-ribosomal fractions. C. Ath-18S rRNA and Ath-U2 in the AGO1-IP.

miR319 targets: TCP3, TCP4, TCP2
miR159 targets: MYB33, MYB65
miR173 targets: TAS1a, TAS1b, TAS2

R: Ribosomal Fraction NR: Non-Ribosomal Fraction T: Total RNA

To test whether in Arabidopsis, miRNA activities, especially miRNA association with ribosomes, change with biological and developmental contexts, we used an anti-AGO1 antibody to perform immunoprecipitation from ribosomal and non-ribosomal fractions in 20 day-old seedlings. As shown in Figure 3.14 A, by doing RT-PCR, we found that when comparing to the dry seeds, in 20 day-old seedlings miRNAs were more strongly associated with ribosomes. Moreover, some miRNA targets were also detected in the AGO1-immunoprecipitates. For example, the miR319 targets, TCP4 and TCP2, were both detected in AGO1-immunoprecipitates from ribosomal and non-ribosomal fractions. The miR159 targets (MYB33 and MYB65), however, were absent in both fractions even though miR159 was obviously present in the AGO1-immunoprecipitates from ribosomal fractions. Because it has been shown that in seedlings and vegetative tissues, miR159 acts to completely silence MYB33/65 transcripts (Allen et al., 2007; Reyes and Chua, 2007; Alonso-Peral et al., 2010), it was not surprising that MYB33/65 transcripts were absent from these fractions. However, the fact that miR159 was present in the ribosomal fractions raised the question of whether or not miR159 in seedlings had other targets that demanded translational control.

Discussion

In animal systems, it has been known that miRNAs mediate the expression of their cognate mRNAs via translational repression (Bartel, 2004; Eulalio et al., 2008b). Mechanistically, it was believed that the major factor underlying this miRNA-imposed translational regulation originates from the nature of the limited base-pairing between animal miRNAs and their targets (Bartel, 2004; Eulalio et al., 2008b; Huntzinger and Izaurralde, 2011). By contrast, because of the much more extensive complementarity between plant miRNAs and targets, plant miRNAs were thought to act predominantly by promoting target cleavage and degradation (Bartel, 2004; Mallory and Bouche, 2008; Huntzinger and Izaurralde, 2011). However, it has now become clear that miRNAs can trigger mRNA degradation in animals (Yekta et al., 2004) and, conversely, translational inhibition in plants (Brodersen et al., 2008). Whether in animals target silencing is achieved predominantly by mRNA degradation or during the steps of translation is still largely under debate. Four distinct mechanisms have been proposed by which miRNAs regulate gene expression: inhibition of translation initiation; inhibition of translation elongation; co-translational protein degradation; and ribosome drop-off (Bartel, 2009; Carthew and Sontheimer, 2009; Voinnet, 2009; Krol et al., 2010a; Huntzinger and Izaurralde, 2011).

In plant systems, although it is now recognized that translational repression is widely present (Brodersen et al., 2008), very little is known at the mechanistic and genetic levels as to what protein effectors are responsible for re-programming the miRISC from directing target cleavage to mediating translational repression. Moreover, because plant miRNAs that

mediate translational repression also cause transcript cleavage (Llave et al., 2002b; Sunkar et al., 2006; Wu and Poethig, 2006; Beauclair et al., 2010), it is difficult to distinguish the relative biological significance of translational inhibition from target degradation. In the current study, we attempted to understand how miRNAs are involved in this translational regulation, and to what extent this mechanism contributes to plant miRNA activities.

To approach this question, by incorporating a Next-Gen sequencing study and RT-qPCR assays, we were able to identify several miRNAs that were preferentially co-localized with actively translating polysomes. Moreover, treating polysomes with puromycin, a chemical widely used to block translation elongation, not only dissociated polysomes, but also released miRNAs that were previously found to be associated with them. These results together suggested that the observed polysomal association of miRNAs was likely to be a product of *bona fide* miRNA-target interactions.

The outcomes of these polysomal miRNA-target interactions could be inhibition of translation elongation, ribosome drop-off, co-translational protein degradation, and/or a combination of all three. We favor the model of miRNA-mediated inhibition of translation elongation, which fits well with experimental data. Take the case of miR319 for example. First of all, our deep sequencing data as well as RT-qPCR results revealed a preferential distribution of miR319 across polysomal fractions. In addition, this polysomal association seemed to be temporally and tissue-independent because in embryos and endosperm harvested at various developmental stages, miR319 consistently exhibited higher abundance

across polysomal fractions in relation to its non-polysomal levels when compared to other miRNAs. The fact that miR319 was found to be strongly associated with polysomes argues against the model where miRISC-miR319 represses target protein synthesis by blocking translation initiation by either competing with eIF4E cap-binding activity or preventing the functional assembly of 80S ribosomes (two possible models for miRNA modes of operation, as discussed in Chapter 1 and earlier in this chapter). We based our arguments on (1) in this "initiation" model, the presence of ribosomes and miRISCs should be mutually exclusive on target mRNAs and (2) in our study, miRISCs could be immunoprecipitated by an antibody against RPL18, a ribosomal protein of 60S subunits (Figure 3.12). Because 60S subunits enter the translation machinery only when the scanning by the 43S complex (40S subunit and associated initiation factor) is completed (reviewed in Jackson, 2010), should blocking of initiation be the only inhibitory mechanism here, miRISCs could not be present in the RPL18- immunoprecipitates. In addition, computational prediction indicated that, in maize, miR319 targets genes encoding proteins including MYB, TCP transcription factors, and ubiquitin carboxyl-terminal hydrolases. As shown in Figure 3.15, miR319 target sites on these three transcripts are located at least 900 nt downstream of their 5' ends. If miR319 represses the protein production via interfering with the elongation step, we should be able to observe certain patterns of miR319 local enrichment across polysomal fractions. Moreover, because of the fairly long distance between translation start sites and miR319 binding sites, multiple ribosomes would have been allowed to be loaded and translocated on the transcripts before being blocked by miRISCs. Therefore, the local enrichment of miR319 probably

would occur in the fractions with large polysomes, which is consistent with our experimental observations.

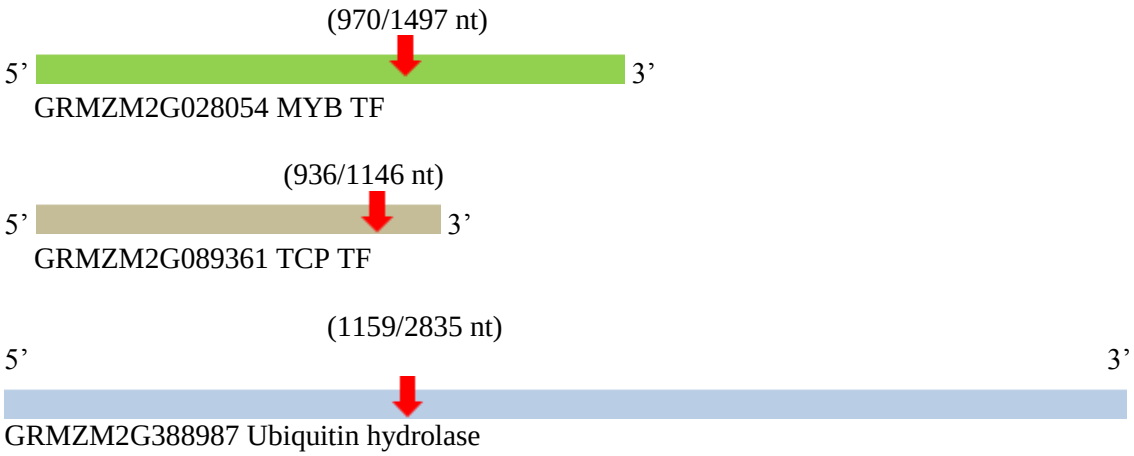


Figure 3.15 Illustration of the miR319 target site on selected miR319 targets.

Arrows indicate the region on transcripts where miR319 target sites reside. Numbers in parentheses indicate the nucleotide position adjacent to target sites in comparison to transcript full length (binding site/full length).

When examining the distribution pattern of miR319 across polysomal fractions, as a general trend we noticed that the relative abundance of miR319 was higher in fractions containing dimer or larger polysomes. However, we couldn’t exclude the possibility of miRNA-induced ribosome drop-off. Although it is tempting to speculate that ribosome drop-off would result

in a local enrichment of miRNAs on smaller polysomes, in this model miRNA distribution patterns are actually combinatorially determined by the rate of ribosome drop-off and the concurrent ribosome loading during translocation.

Additional evidence supporting the miRNA-mediated repression of translation elongation arose from our immunoprecipitation studies. By exploiting the 35S-HF-*RPL18* transgenic system, we were able to perform immunoprecipitation using anti-FLAG immobilized beads. Both the miRNA core effector AGO1 and miRNAs were detected in the anti-FLAG immunoprecipitates. Reciprocally, when immunoprecipitation was carried out using anti-AGO1 antibody in the same transgenic system, FLAG-tagged ribosomal protein RPL18 was detected in the corresponding immunoprecipitates (data not shown). Additionally, when doing immunoprecipitation experiments using anti-AGO1 antibodies in both ribosomal and non-ribosomal fractions, we found that in dry Arabidopsis seeds, miRNAs were predominantly detected in non-ribosomal fractions. However, in imbibed Arabidopsis seeds and 20-day seedlings, many of the miRNAs displayed higher abundance in ribosomal fractions. These findings could be a reflection of the significant role of miRNA-mediated translational repression in modulating various biological programs. As dry seeds undergo imbibition, they quickly resume active physiology such as energy metabolism (reviewed in Rajjou et al., 2012). Consequently, many stored mRNAs re-enter the translational machinery to produce proteins needed to adapt to such biological processes. In fact, it has been shown that although the speed and uniformity of germination were greatly affected, inhibition of gene transcription by a potent RNA POL II inhibitor α-amanitin failed to abolish radicle

protrusion through seed coats. On the contrary, inhibiting protein synthesis by cycloheximide completely blocked the germination of Arabidopsis and rice seeds (Dure and Waters, 1965; Rajjou et al., 2004; He et al., 2011). Because resumption of the translation of stored mRNAs is essential for proper seed germination, it is reasonable to postulate that some miRNAs detected in dry seeds will resume exerting their roles to control the target translation following imbibition. As expected, in the immunoprecipitates pulled down by anti-FLAG antibody from 24-hr-imbibed *35S-FLAG-RPL18* transgenic seeds, we found that AGO1 as well as many miRNAs were associated with functional ribosomes. An even more dramatic change was observed in 20-day seedlings. By doing a pull-down study with anti-AGO1 antibody, we showed that miRNAs and some miRNA targets co-precipitated with AGO1, and interestingly, a large portion of the miRNAs were found in ribosomal fractions. Taken together, the finding that there was a shift in the association of miRNAs with the translational machinery from physiologically inert dry seeds to developing seedlings suggested that miRNA-mediated translational control very likely echoed the demands for modulation of various biological events leading to normal plant development (summarized in Figure 3.16).

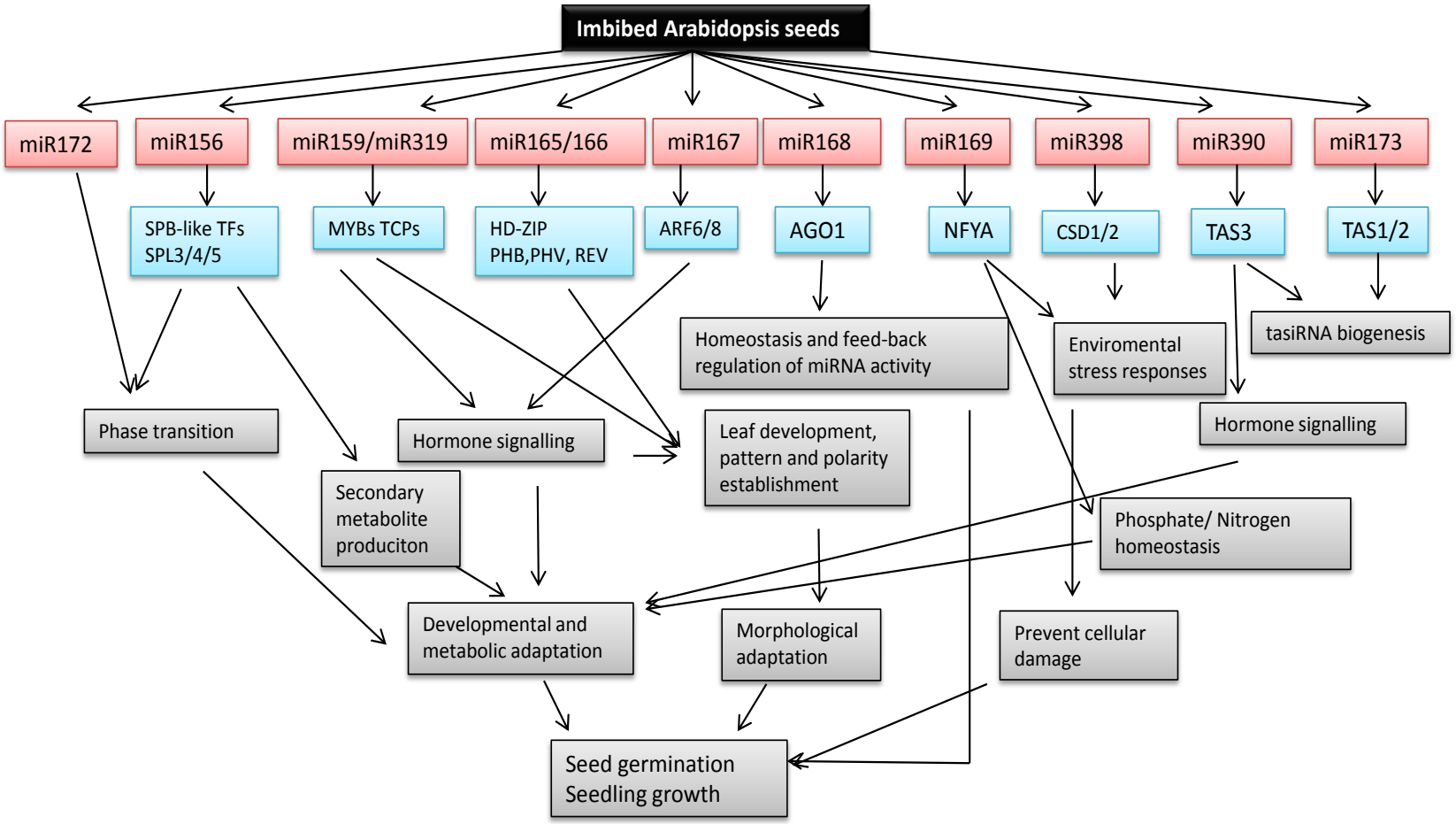


Figure 3.16 The potential regulatory network for miRNAs during and post Arabidopsis seed germination.

miRNAs shown in red boxes represent the miRNA families present in AGO1-immunopreicipitates from Arabidopsis dry seeds and 20-day seedlings. Targets of miRNAs are shown in blue boxes. Biological processes potentially regulated by various miRNAs are shown in grey boxes.

Our data indicated that despite various degrees of preference, during seed development and germination, miRNAs were associated with actively translating polysomes. This finding raised several interesting thoughts. First of all, because (1) the degree of polysomal association of miRNAs appeared to be species-specific and (2) the relative abundance of miRNAs found in polysomal fractions did not always correlate with their overall abundance, there must be a selection mechanism to determine which miRNAs were to be recruited to polysomes. Moreover, the recruitment of miRNAs to polysomes did not result from substitution of the miRISC effector AGO1 with other AGO homologs, as immunoprecipitations by the anti-AGO1 antibody were able to successfully pull down miRNAs from both ribosomal and non-ribosomal fractions. The GW-repeat protein SUO in Arabidopsis has been suggested to interact with AGO1 to guide miRNA-mediated translational repression events (Yang et al., 2012). However, we reasoned that SUO was not likely to be the factor responsible for linking miRISCs to the translation machinery because (1) the subcellular localization of SUO protein overlapped with the cytoplasmic foci known to be P bodies (Yang et al., 2012) and (2) P bodies have been shown to be depleted of ribosomal components and activity (Brengues et al., 2005; Teixeira et al., 2005; Parker and Sheth, 2007). In addition, even though our data were suggestive that the miRNA-mediated translational control was dynamic and possibly involved in a variety of physiological events throughout the plant life cycle, evidence was insufficient to address the question as to whether this seemingly miRNA-specific regulatory mechanism *per se* was regulated in a temporally or in a tissue- specific manner. Results from our maize germination study provide some evidence to answer this question. By doing miRNA semi-quantitative RT-PCR,

we showed that for certain miRNAs, such as miR319 and miR390, their association with ribosomes differed between endosperm and embryos, and these polysomal distribution patterns were also changing as seed germination progressed. Last but not least, how significantly does this translational control contribute to overall miRNA activities? Or more fundamentally, what kind of evolutionary pressure entailed plants to acquire such a regulatory mechanism? The latter one might be easier to answer: Plants are immotile; therefore by nature they are required to produce more agile responses to environmental and developmental cues. Translational control would be a cost-effective and swift measure plants could employ to make quick physiological adaptation to rapidly changing environments. Furthermore, many biological processes such as developmental phase transitions require transient regulation of gene expression. Translational modulation rather than transcriptional regulation is more advantageous in these scenarios because transcripts that are temporarily arrested for translation can be stored in cellular compartments and re-enter the translation machinery later (Brengues et al., 2005; Parker and Sheth, 2007; Balagopal and Parker, 2009). Therefore, instead of acting as a "switch" to completely silence target gene expression, miRNA-mediated translational control can potentially function as a "tuner", to finely and rapidly modulate gene expression to meet various metabolic and physiological needs during plant development and stress responses.

Although our data are in agreement with those from a previous study, indicating translational repression is a widespread mode of operation for plant miRNAs (Brodersen et al., 2008), many aspects of this mechanism remain unknown. Although some mechanistic similarities

might be present between animal and plant miRNA-mediated translational repression, as suggested by the requirement for microtubule networks and P-body components in both systems, one of the obstacles hindering the understanding of miRNA-directed repression in plants is our poor knowledge of AGO1 interacting proteins. In animal systems, several proteins including the GW-rich protein GW182 and the ribosomal protein RACK1 (Receptor for Activated-C Kinase 1) have been characterized as AGO-interacting and essential for miRNA activities (Meister et al., 2005; Eulalio et al., 2008a; Jannot et al., 2011). Particularly, the finding that RACK1 interacts with AGO1 and is required for miRNA function in yeast and mammalian cells calls for attention because RACK1 is a constituent of the 40S ribosomal subunit (Jannot et al., 2011; Otsuka et al., 2011; Gibson, 2012), and is conserved among different species. In Arabidopsis, there are 3 RACK1 homologs, namely RACK1A, RACK1B and RACK1C (Guo and Chen, 2008). Interestingly, Guo et al. reported that RACK1 acted to negatively regulate ABA responses, as *rack1* mutants exhibited hypersensitivity to ABA treatment (Guo et al., 2009). Later, the role of RACK1 as a negative regulator of ABA responses was supported by the finding that it facilitated 80S ribosome assembly and protein translation (Guo et al., 2011b). In this study, Guo et al. demonstrated a physical interaction between RACK1 and the eukaryotic translation initiation factor eIF6 (Guo et al., 2011b). Based on the data, they proposed that RACK1 was involved in triggering the phosphorylation of eIF6, thereby facilitating the assembly of 60S and 40S ribosomal subunits to form functional 80S ribosomes (Guo et al., 2011b).

Even more interestingly, and perhaps not coincidentally, evidence has shown that eIF6 was involved in miRNA activities in human cell lines and *C. elegans*. In this study, depletion of eIF6 abrogated miRNA-directed regulation of target protein synthesis and mRNA accumulation (Chendrimada et al., 2007). By examining proteins immunoprecipitated with the minimal miRISCs (AGO2-TRBP-DICER), Chendrimada et al. found that most of the components of ribosomal 60S subunits as well as eIF6 were present in the complexes associated with miRISCs (Chendrimada et al., 2007). Surprisingly, no constituent of the ribosome 40S subunit was found in the immunoprecipitates (Chendrimada et al., 2007). Based on these findings, Chendrimada et al. proposed a model where by competing with the translational machinery for 60S subunits and the 60S subunit-associated protein eIF6, miRNAs acted to block the translation initiation, and hence repress the target translation (Chendrimada et al., 2007). However, one cannot exclude the possibility that the large complexes of the 60S subunit, eIF6 and miRISC also are involved elsewhere in the miRNA functional pathway. We will develop this argument in our proposed model for plant miRNA-mediated translational regulation.

In Arabidopsis, biochemical evidence demonstrated that the loading of miRNAs into AGO1 was facilitated by HSP90 (Iki et al., 2010). Interestingly, in animal systems, similar observations were made (Iwasaki et al., 2010). It was proposed that HSP90 was responsible for triggering a conformational change of AGO proteins such that they can receive bulky, rigid small RNA duplexes (Iwasaki et al., 2010; Kawamata and Tomari, 2010). Moreover, a plant cyclophilin 40 (CyP40; SQN) was also reported to be required for miRNA activity in

Arabidopsis (Wu et al., 2009). In yeast (Duina et al., 1996) and mammals (Hoffmann and Handschumacher, 1995; Freeman et al., 1996; Ratajczak and Carrello, 1996; Pirkl and Buchner, 2001), CyP40 has been shown to harbor both peptidylprolyl isomerase (PPIase) and protein chaperone activity and is thought to act in association with HSP90. In plants, CyP40 was also reported to be associated with HSP90 (Owens-Grillo et al., 1996). However, although HSP90 and CyP40 are both involved in the miRNA functional pathway in plants, their roles cannot be completely overlapping because, firstly, *hsp90* and *sqn/cyp40* mutants manifested phenotypes that only partially resembled each other, and secondly, miRNA abundance was affected in *hsp90* while remaining unchanged in *sqn/cyp40* plants (Wu et al., 2009). This finding is in line with the previous discovery that HSP90 in plants facilitates the loading of miRNAs into miRISCs (Iki et al., 2010). Moreover, in animals, HSP90 not only was reported to facilitate the nuclear export of ribosomal 60S subunits (Schlatter et al., 2002), but also played a role in regulating the function of ribosomes by maintaining the stability of 40S ribosomal subunit proteins such as S3 and S6 (Kim et al., 2006). Kim et al. argued that, by directly interacting with these ribosomal proteins, HSP90 prevented these ribosomal proteins from being ubiquitinated and degraded via the 26S proteasome protein degradation pathway (Kim et al., 2006).

Importins have also been shown to be involved in the miRNA functional pathway. In Arabidopsis, Qi et al. characterized an importin β as a negative regulator of miRNA activity via controlling miRNA loading into miRISCs (Wang et al., 2011b). In human cells, importin 8 (Imp8) interacts with AGO proteins and localizes to P bodies. Interestingly, in this case,

instead of being a negative regulator, Imp8 was shown to be required for the recruitment of AGO protein complexes to target mRNAs, allowing efficient and specific gene silencing (Weinmann et al., 2009).

The proposed model for plant miRNA-mediated translational repression

As aforementioned, in both plant and animal systems, HSP90 physically interacts with AGO1, and presumably changes the conformation of AGO1 to facilitate the incorporation of miRNAs into the small RNA binding pocket harbored at the protein core (Iki et al., 2010; Iwasaki et al., 2010). Moreover, at least in animals, HSP90 is involved both in maintaining the normal function of ribosomes via directly interacting with 40S subunit proteins (Kim et al., 2006), and shuttling 60S subunits from the nucleus into the cytoplasm (Schlatter et al., 2002). Intriguingly, in animals, the ribosomal 40S subunit-associated protein RACK1 and the 60S subunit constituent eIF6 have both been shown to be essential parts of the miRNA functional pathway (Chendrimada et al., 2007; Jannot et al., 2011). Even though to date no direct experimental evidence has linked the functions of plant RACK1 and eIF6 to miRNA activities, reports have been made that RACK1 regulates multiple hormonal responses and developmental processes in Arabidopsis (Chen et al., 2006; Guo and Chen, 2008). In addition, a previous study specifically pointed out the involvement of RACK1 in ABA-induced signal transduction by showing that *rack1* mutants exhibited hypersensitivity to ABA treatment (Guo et al., 2009). It has long been known that plant miRNAs play essential roles in modulating hormonal responses (reviewed in Zhang et al., 2006; Voinnet, 2009), and several miRNAs have been shown to be regulators of ABA signaling networks (Liu et al.,

2007; Reyes and Chua, 2007; Jia et al., 2009). The finding that RACK1 and many miRNAs share domains of expression and function suggests a potential role of RACK1 in many miRNA activities. Therefore, it is plausible to infer from the animal system to speculate that RACK1 and eIF6 are also essential for miRNA activities in plants.

Based on the discussion above and our experimental data, in Figure 3.17 we hereby propose a model for plant miRNA-mediated translational repression. Even though in plants a preponderance of evidence supports the model that miRNA-mediated translational control occurs in a post-initiation manner, we cannot formally exclude the possibility that plant miRNAs also contribute to translational repression via blocking translation initiation. To serve the purpose of our discussion, only a model that fits the post-initiation theory is presented (Figure 3.17).

As shown in Figure 3.17, in the cytoplasm, AGO1 binds to the small RNA duplex in collaboration with HSP90 in an ATP-dependent manner **[step A]** (Iki et al., 2010; Iwasaki et al., 2010). It is believed that the presence of HSP90 causes a conformational change of AGO1 to better accommodate the small RNA duplex in the binding pocket (Iwasaki et al., 2010; Landthaler, 2010). During maturation of miRISCs, eIF6, the 60S ribosomal subunit, and possibly CyP40 join the miRISC-HSP90 complex to form a larger protein complex **[step B]**. This complex then is recruited to cytosolic polysomes possibly via the activity of ribosomal 60S subunits **[step C]**. Once the big complex is joined by the 40S ribosomal subunit on polysomes, eIF6 is phosphorylated and dissociated from the complex **[step 1.1]**.

In mammals, the phosphorylation of eIF6 is an outcome of the activity of protein kinase C (PKC), which is recruited to ribosomes by the 40S ribosomal protein RACK1 (Nilsson et al., 2004; Adams et al.,2011; Guo et al.,2011a; Gibson,2012). The release of eIF6 from 60S subunits promotes the assembly of 80S ribosomes **[step 1.2]** (Ceci et al., 2003). During the 80S ribosome assembly, possibly guided by HSP90, miRISC is anchored on RACK1, where a potential plant PKC-like kinase phosphorylates AGO1 **[step 1.3]**. In animals, phosphorylation of certain serine and tyrosine residues on AGO proteins has been shown to change the AGO protein activity and subcellular localization (Rudel and Meister, 2008; Zeng et al., 2008; Rudel et al., 2011). Such phosphorylation of AGO1 can potentially also change the AGO1 slicing activity. Therefore instead of causing cleavage of target mRNAs, when phosphorylated, AGO1 would function as a translational repressor **[step 2]**. Because in this proposed model, miRISCs are associated with ribosomes, the deposition of the miRISC to the cognate site would be fulfilled simply by the ribosome translocation along mRNAs **[step 3].** Once the ribosome and associated-miRISC complex move to the proximity of the miRNA target site, phosphorylated AGO1 would bind to the site by base-pairing **[step 4]** and start exerting its role to repress translation via either blocking the elongation process **[step I]** or causing the ribosome drop-off **[step II]**.

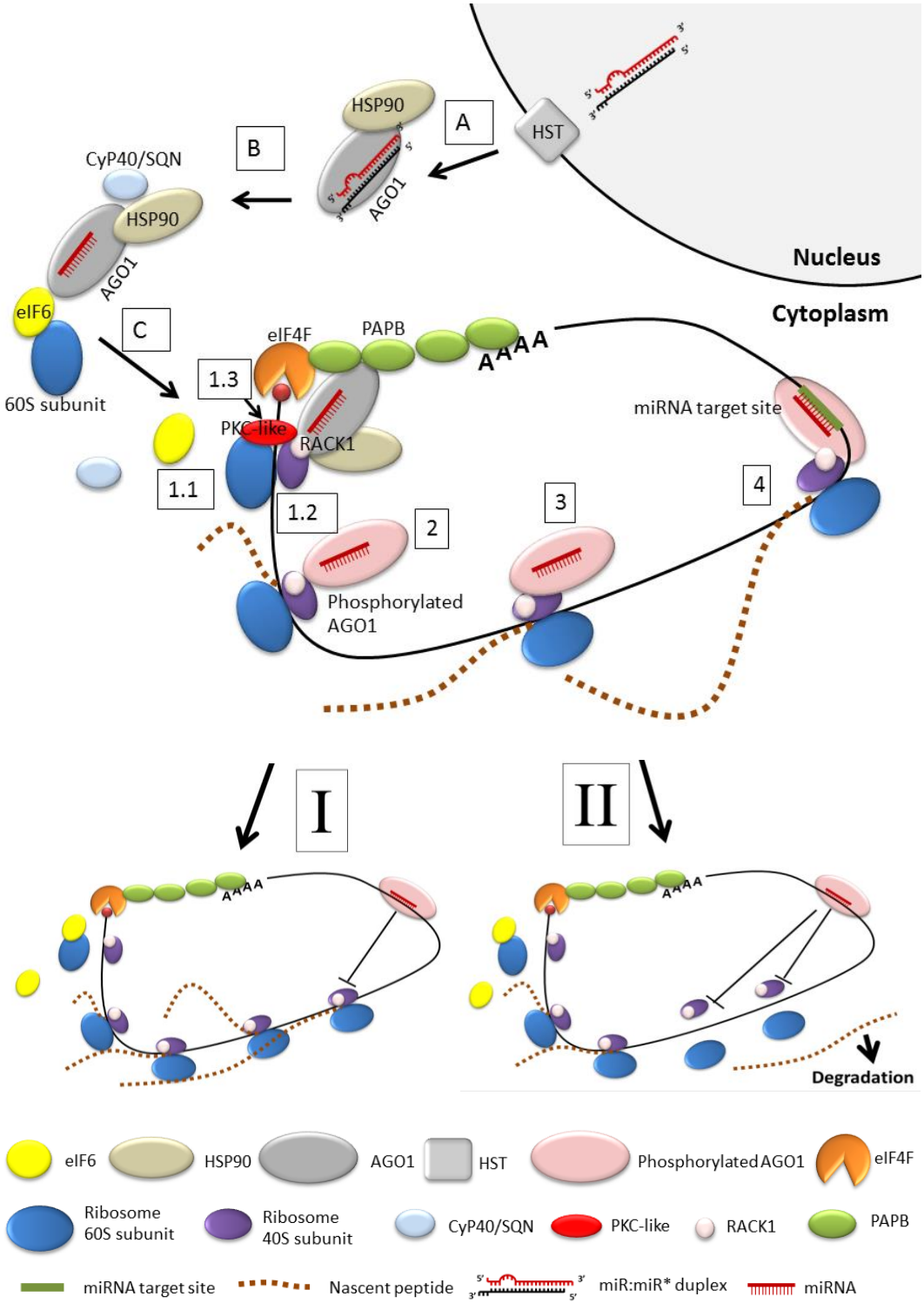
Although mechanistically this model allows us to reconcile the existing data on plant miRNA-mediated translational repression, many additional factors have been shown to be involved in this translational control. As aforementioned, in Arabidopsis, the AGO1 homolog AGO10, decapping complex components DCP1, DCP2 and VCS, and the microtubule

severing factor KTN are all involved in this mechanism (Brodersen et al., 2008; Motomura et al., 2012). In the following part, we will discuss the mode of miRNA actions from various perspectives.

Figure 3.17 Model for plant miRNA-mediated translational repression.

After the miR;miR* duplex is exported out of the nucleus by HST, AGO1 binds to the small RNA duplex in collaboration with HSP90 to form the HSP90-miRISC complex. The HSP90-miRISC complex becomes larger upon the binding of eIF6, the 60S ribosomal subunit, and possibly CyP40. This larger complex then is recruited to cytosolic polysomes, where eIF6 is phosphorylated and released from the complex. The release of eIF6 from 60S subunits promotes the assembly of 80S ribosomes. Meanwhile, possibly guided by HSP90, miRISC interacts with RACK1, which recruits PKC-like to phosphorylate AGO1, and moves along with ribosomes. Once the ribosome and the associated-miRISC move to the proximity of the miRNA target site, miRISC binds to it by base-pairing and starts exerting its role to repress translation via either blocking the elongation process or causing the ribosome drop-off. Phosphorylation potentially changes the slicing activity of AGO1, therefore, instead of causing the target cleavage, AGO1 on polysomes only directs translational inhibition.

HST: Exportin5-homolog HASTY; PAPB: Poly-A Binding Protein; RACK1: Receptor for Activated Kinase C; PKC-like: Protein Kinase C-like


Nucleus
Cytoplasm
HST
HSP90
AGO1
A
B
CyP40/SQN
HSP90
AGO1
eIF6
60S subunit
C
eIF4F
PAPB
AAAA
1.3
PKC-like
RACK1
1.1
1.2
2
3
4
miRNA target site
Phosphorylated AGO1
I
II
Degradation
eIF6
HSP90
AGO1
HST
Phosphorylated AGO1
eIF4F
Ribosome 60S subunit
Ribosome 40S subunit
CyP40/SQN
PKC-like
RACK1
PAPB
miRNA target site
Nascent peptide
miR:miR* duplex
miRNA

Phosphorylation: A switch that governs the multifaceted role of AGO1?

In our model, we proposed that phosphorylation might affect the slicing activity of AGO1. Particularly, if the phosphorylation occurs on residues located in proximity to the AGO1 catalytic domain, it may potentially interfere with the RNase H enzymatic activity of AGO1, rendering it no longer able to carry out endonucleolytic cleavage of target transcripts.

In an effort to support our model, we surveyed several plant protein phosphorylation databases (Heazlewood et al., 2008; Gao et al., 2009; Yao et al., 2012; Zulawski et al., 2013; S. Briggs, personal communication of manuscript in preparation: Walley, J., Shen, Z., Sator, RC., Wu, KJ., Osborn, LG., Briggs, SP. Reconstruction of Protein Networks from an Atlas of Proteotypes.) to look for evidence supporting that plant AGO1 *in vivo* indeed can be phosphorylated. By comparing detected phosphorylated peptides derived from maize, rice and Arabidopsis AGO1 proteins, we found a motif within which certain serine and threonine residues were phosphorylated. Sequence analyses revealed that this phosphorylated motif resided in the PIWI domain, and intriguingly, it was located close to the histidine residue of the "DDH" catalytic site. As shown in Figure 3.18, the yellow boxed residues are the reported catalytic sites responsible for AGO1's endonucleolytic slicing activity (Song et al., 2004; Baumberger and Baulcombe, 2005; Rivas et al., 2005; Yuan et al., 2005; Kapoor et al., 2008; Qian et al., 2011). We found that a serine residue (Ath-AGO1 S1001), located 14-residues to the C-terminal side of the catalytic amino acid D986, frequently underwent phosphorylation. All of the maize AGO1 orthologs (except for Zma-AGO1d, which was not found in the maize proteomics database), Arabidopsis AGO1, as well as two rice AGO1

orthologs (out of a total of 4 Osa-AGO1 genes) were phosphorylated at the same serine site. Moreover, some serine and threonine residues in the surrounding area were also often shown to be phosphorylated. Notably, Ath-AGO10, the closest Ath-AGO1 homolog, does not display a sequence consensus in this area, and no such phosphorylation was detected. Interestingly, computational protein surface accessibility analysis (Petersen et al., 2009) revealed that S1001 in the Ath-AGO1 protein was characterized as an "exposed residue"(Figure S5). This finding is in line with the idea that phosphorylation sites are typically located on the surface of proteins to allow for efficient access by kinases and phosphatases.

It is worth noting that phosphorylation of this serine residue (Zma-AGO1a S1054; Ath-AGO1 S1001) doesn't seem to occur on all AGO1 proteins. As shown in Figure 3.19, instead of being ubiquitously phosphorylated (or non-phosphorylated), Zma-AGO1 proteins exist in both phosphorylated and non-phosphorylated forms in various tissue types. We propose that this phosphorylation works to modulate the AGO1 activity. As discussed in my model, phosphorylation of the conserved serine residue inhibits the AGO1 slicing activity, possible by interfering with the hydrolysis of mRNA substrates facilitated by the "DDH" domain. The phosphorylated AGO1 acts as a translational repressor to modulate the protein production of target genes. Whether the phosphorylation occurs only on polysomes is still an open question. Nevertheless, our model suggests that phosphorylation may be the post-translational modification that determines whether AGO1 will act to cleave target transcripts, or to modulate the gene expression at the level of protein translation.

Because the phosphorylated serine residue seems to be conserved among plant AGO1 proteins, one might reason that instead of inhibiting the AGO1 slicing activity, this particular phosphorylation might be essential for regular AGO1 functions. However, we argue against that possibility for the following reasons: (1) the AGO7 protein is one of the three AGOs (AGO1, 4, and 7) in Arabidopsis that has demonstrated slicing activities (reviewed in Vaucheret, 2008). If a phosphorylation site analogous to S1001 on Ath-AGO1 is crucial for the slicing activity of AGO7, a consensus S or T residue should be observed. However, we couldn't identify such a phosphorylation site in the C terminal regions of Ath-AGO7, Zma-AGO7 or Osa-AGO7, due to the poor sequence homology among them (Figure S6). In addition, we couldn't find any phosphorylated peptides from AGO7 in any of the protein phosphorylation databases we surveyed. (2) To date, human Hsa-AGO2, Drosophila Dme-AGO1, Dme-AGO2, Arabidopsis Ath-AGO1, Ath-AGO4, and Ath-AGO7 are the only AGO proteins that have experimentally verified slicing activities. It has been suggested that the structural characteristics of AGOs contributing to the slicing activity are well conserved between the animal and plant kingdoms (Baumberger and Baulcombe, 2005). Therefore we performed a multiple sequence alignment using the abovementioned AGO protein sequences. Expectedly, we found the conserved "DDH" domain in all AGOs. However, no apparent sequence homology or potential phosphorylation site was found in the region beyond the catalytic H residue (Figure S7). The absence of a consensus phosphorylation motif among these slicer AGOs again suggested that phosphorylation was dispensable for the AGO slicing activity. (3) Bacterially expressed recombinant human AGO2 protein exhibited slicing activity in an *in vitro* system (Rivas et al., 2005). Because to date, no AGO homologs have

been found in prokaryotes, recombinant Hsa-AGO2 protein expressed in *E.coli* was not likely to be phosphorylated, if at all, in the same manner as it would be in eukaryotic cells. Therefore the demonstrated slicing activity didn't seem to be dependent on proper phosphorylation. Taken together, since the slicing activity of AGO proteins seems to be independent of a conserved phosphorylation site, we favor the model where phosphorylation of AGO1 prevents it from directing transcript cleavages, and allows a regulatory mechanism by which AGO1acts to translationally modulate protein synthesis, without drastically affecting the target mRNA pools.

Figure 3.18 Mulitiple alignments of protein sequences within the PIWI domain of AGO1 proteins from maize, rice and Arabidopsis.

The yellow boxed residues are the reported catalytic sites responsible for the AGO1 slicing activity. The green highlighted residues are the phosphorylated sites. A serine (boxed) 14-residues to the C-terminal side of the histidine in the "DDH" domain was found to be phosphorylated in all detected Zma-AGO1s, Ath-AGO1 and some Osa-AGO1s. In the case of Zma-AGO1b, even though no localized phosphorylation sites were detected, analyses of phosphorylated peptides indicated that some T and S residues (Blue highlighted) had more than 50% probability of being phosphorylated.

PIWI Domain

```
OsAGO1c          IGILPDNNGSLYGDLKRVCEIDLGIVSQCCCTKQVFKMNK-QILANLALKINVKVGGRNT
ZmAGO1d          IGILPDNNGSLYGDLKRICEIDLGLVSQCCCAKQVFKMNK-QILANLALKINVKVGGRNT
OsAGO1d          IGLLPDNNGSLYGDLKRICEIDLGLVSQCCCTKQVFKMNK-QILANLALKINVKVGGRNT
AtAGO10          LAILPDNNGSLYGDLKRICETELGLISQCCLTKHVFKISK-QYLANVSLKINVKMGGRNT
AtAGO1           IVILPDNNGSLYGDLKRICETELGIVSQCCLTKHVFKMSK-QYMANVALKINVKVGGRNT
OsAGO1a          IAILPDNNGSLYGDLKRICETDLGLVSQCCLTKHVFKMSK-QYLANVALKINVKVGGRNT
OsAGO1b          IVILPDNNGSLYGDLKRICETDLGLVSQCCLTKHVFKMSK-QYLANVALKINVKVGGRNT
ZmAGO1b          IVILPDNNGSLYGDLKRICETELGLVSQCCLTKHVFKMSK-QYLANVALKINVKVGGRNT
ZmAGO1c          IVILPDINGSLYGDLKRICETDLGLVSQCCLTKHVFKMSK-QYLANVALKINVKVGGRNT
ZmAGO1a          MVILPDNNGSLYGDLKRICETDLGLVSQCCLTKHVFKANKHQYLANVALKINVKVGGRNT
ZmAGO1e          IVILPDNNGSLYGDVKRICETNLGLVSQCCLTKHVFKVNKQQYLANVALKINVKVGGRNT
                 : :*** *******:**:** :**::**** :*:*** .* * :**::******:*****

OsAGO1c          VLVDAVSRRIPLVTDRPTIIFGADVTHPHPGEDSSPSIAAVVASQDWPEVTKYAGLVSAQ
ZmAGO1d          VLADAVSRRIPLVTDRPTIIFGADVTHPHPGEDSSPSIAAVVASQDWPEVTKYAGLVSAQ
OsAGO1d          VLVDAVSRRIPLVTDRPTIIFGADVTHPHPGEDSSPSIAAVVASQDWPEVTKYAGLVSAQ
AtAGO10          VLVDAISCRIPLVSDIPTIIFGADVTHPENGEESSPSIAAVVASQDWPEVTKYAGLVCAQ
AtAGO1           VLVDALSRRIPLVSDRPTIIFGADVTHPHPGEDSSPSIAAVVASQDWPEITKYAGLVCAQ
OsAGO1a          VLVDALTRRIPLVSDRPTIIFGADVTHPHPGEDSSPSIAAVVASQDWPEVTKYAGLVSAQ
OsAGO1b          VLVDALTRRIPLVSDRPTIIFGADVTHPHPGEDSSPSIAAVVASQDWPEVTKYAGLVSAQ
ZmAGO1b          VLLDALSRRIPLVSDRPTIIFGADVTHPHPGEDSSPSIAAVVASQDWPEVTKYAGLVSAQ
ZmAGO1c          VLVDALTRRIPLVSDRPTIIFGADVTHPHPGEDSSPSIAAVVASQDWPEVTKYAGLVSAQ
ZmAGO1a          VLVDALARRIPLVSDVATIIFGADVTHPHPGEDSSPSIAAVVASQDWPEVTKYAGLVSAQ
ZmAGO1e          VLVDALARRIPLVSDIATIIFGADVTHPHPGEDSSPSIAAVVASQDWPEVTKYAGLVSAQ
                 ** **:: *****:* .********** **:****************:*******.**

OsAGO1c          AHRQELIEDLYKIWQDPQRGTVSGGMIRELLISFKRSTGEKPQRIIFYRDGVSEGQFYQV
ZmAGO1d          SHRQELIEDLYNVTHDPQKGTVCGGMVRELLISFKKSTGQKPQRILFYRDGVSEGQFYQV
OsAGO1d          SHRQELIDDLYNITHDPHRGPICGGMVRELLISFKRSTGQKPQRIIFYRDGVSEGQFYQV
AtAGO10          AHRQELIQDLYKTWQDPVRGTVSGGMIRDLLISFRKATGQKPLRIIFYRDGVSEGQFYQV
AtAGO1           AHRQELIQDLFKEWKDPQKGVVTGGMIKELLIAFRRSTGHKPLRIIFYRDGVSEGQFYQV
OsAGO1a          AHRQELIQDLFKVWKDPQRGTVSGGMIRELLISFKRATGQKPQRIIFYRDGVSEGQFYQV
OsAGO1b          AHRQELIQDLFKVWQDPHRGTVTGGMIKELLISFKRATGQKPQRIIFYRDGVSEGQFYQV
ZmAGO1b          AHRQELIQDLFKVWQDPQRRTGTSHFFQEG--NWTEAPEDHILQFLSFRDGVSEGQFYQV
ZmAGO1c          AHRQELIQDLFKVWQDPQRRTVTGGMIKELLISFKRATGQKPQRIIFYRDGVSEGQFYQV
ZmAGO1a          THRQELIQDLFNVRQDPQRGAVSGGMIRELLISFWRATGQKPKRIIFYRDGVSEGQFYQV
ZmAGO1e          AHRQELIQDLFKVWQDPERGTVSGGMIRELLISFWRATGQKPKRIIFYRDGVSEGQFYQV
                 :******:**::  :** .    . :..:    :  :.  :  .:: :************

OsAGO1c          LLYELNAIRKACASLETNYQPKVTFIVVQKRHHTRLFAHNHNDQNSVDRSGNILPGTVVD
ZmAGO1d          LLHELDAIRKACASLEANYQPQVTFIVVQKRHHTRLFAHNHNDQNSVDRSGNILPGTVVD
OsAGO1d          LLHELDAIRKACASLEANYQPQVTFIVVQKRHHTRLFAHNHNDQNSVDRSGNILPGTVVD
AtAGO10          LLYELDAIRKACASLEPNYQPPVTFIVVQKRHHTRLFANNHRDKNSTDRSGNILPGTVVD
AtAGO1           LLYELDAIRKACASLEAGYQPPVTFVVVQKRHHTRLFAQNHNDRHSVDRSGNILPGTVVD
OsAGO1a          LFYELDAIRKACASLEADYQPPVTFVVVQKRHHTRLFANNHKDQRTVDRSGNILPGTVVD
OsAGO1b          LLYELDAIRKACASLEPNYQPPVTFVVVQKRHHTRLFANNHNDQRTVDRSGNILPGTVVD
ZmAGO1b          LLYELDAIRKACASLEPNYQPPVTFVVVQKRHHTRLFANNHSDQRTVDRSGNILPGTVVD
ZmAGO1c          LLYELDAIRKACASLEPNYQPPVTFVVVQKRHHTRLFANNHNDQRTVDRSGNILPGTVVD
ZmAGO1a          LLYELDAIRKACASLESDYQPPVTFVVVQKRHHTRLFVNNHNDQRAADRSGNILPGTVVD
ZmAGO1e          LLYELDAIRKACASLESDYQPPVTFVVVQKRHHTRLFANNHNDNRAVDKSGNILPGTVVD
                 *::**:**********..*** ***:***********.:** *..:.*.***********
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OsAGO1c SKICHPTEFDFYLCSHAGIKGTSRPAHYHVLWDENNFTADALQILTNNLCYTYARCTRSV
ZmAGO1d SKICHPTEFDFFLCSHAGIKGTSRPAHYHVLWDENNFTADALQTLTNNLCYTYARCTRSV
OsAGO1d SKICHPTEFDFFLCSHAGIKGTSRPAHYHVLWDENNFTADALQTLTNNLCYTYARCTRSV
AtAGO10 TKICHPTEFDFYLCSHAGIQGTSRPAHYHVLWDENNFTADGIQSLTNNLCYTYARCTRSV
AtAGO1 SKICHPTEFDFYLCSHAGIQGTSRPAHYHVLWDENNFTADGLQSLTNNLCYTYARCTRSV
OsAGO1a SKICHPTEFDFYLCSHAGIQGTSRPAHYHVLWDENKFTADGLQTLTNNLCYTYARCTRSV
OsAGO1b SKICHPTEFDFYLCSHAGIQGTSRPAHYHVLWDENKFTADELQTLTNNLCYTYARCTRSV
ZmAGO1b SKICHPTEFDFYLCSHAGIQGTSRPAHYHVLWDENKFTADELQTLTNNLCYTYARCTRSV
ZmAGO1c SKICHPTEFDFYLCSHAGIQGTSRPAHYHVLWDENKFTADELQTLTNNLCYTYARCTRSV
ZmAGO1a SKICHPTEFDFYLCSHAGIQGTSRPAHYHVLWDENKFTADGLQTLTNNLCYTYARCTRSV
ZmAGO1e SKICHPTEFDFYLCSHAGIQGTSRPAHYHVLWDENNFTADGLQTLTNNLCYTYARCTRSV
OsAGO1c SIVPPAYYAHLAAFRARFYMEPD-TSDSSSVVSGP--GVRGPLSGSSTSR-TRAPGGAAV
ZmAGO1d SIVPPAYYAHLAAFRARFYMEPD-SSDSGSLASGA--RGGGAPSSSSTSRSTRATAGGAV
OsAGO1d SIVPPAYYAHLAAFRARFYMESD-SSDSGSMASG-----RGG--GSSTSRSTRAAGGGAV
AtAGO10 SIVPPAYYAHLAAFRARFYLEPEIMQDNGS----------PGKKNTK-TTTVGDVGV
AtAGO1 SIVPPAYYAHLAAFRARFYMEPE-TSDSGSMASGS--MARGGGMAGRSTR--GPNVNAAV
OsAGO1a SIVPPAYYAHLAAFRARFYMEPD-TSDSGSMASGAHTRGGGPLPGARSTK---PAGNVAV
OsAGO1b SIVPPAYYAHLAAFRARFYMEPE-TSDSGSMASGA-ATSRGLPPGVRSAR---VAGNVAV
ZmAGO1b SIVPPAYYAHLAAFRARFYMEPD-TSDSGSLASGA----RGPPPGAARSS-TRGAGSVEV
ZmAGO1c SIVPPAYYAHLAAFRARFYMEPD-TSDSGSMASGA----RGPPPGAARSM--RGAGSVAV
ZmAGO1a SIVPPAYYAHLAAFRARFYMEPD-TSDSGSVASGA-TTSRGPPPGARNTR--AGAANVAV
ZmAGO1e SIVPPAYYAHLAAFRARFYMEPD-TSDSGSMAS------RGPPPGGRNTK-AAGVGNVAV
OsAGO1c KPLPALKDSVKRVMFYC
ZmAGO1d RPLPALKDSVKKVMFYC
OsAGO1d RPLPALKDSVKNVMFYC
AtAGO10 KPLPALKENVKRVMFYC
AtAGO1 RPLPALKENVKRVMFYC
OsAGO1a RPLPDLKENVKRVMFYC
OsAGO1b RPLPALKENVKRVMFYC
ZmAGO1b RPLPALKENVKRVMFYC
ZmAGO1c RPLPALKENVKRVMFYC
ZmAGO1a RPLPALKENVKRVMFYC
ZmAGO1e RPLPALKENVKRVMFYC

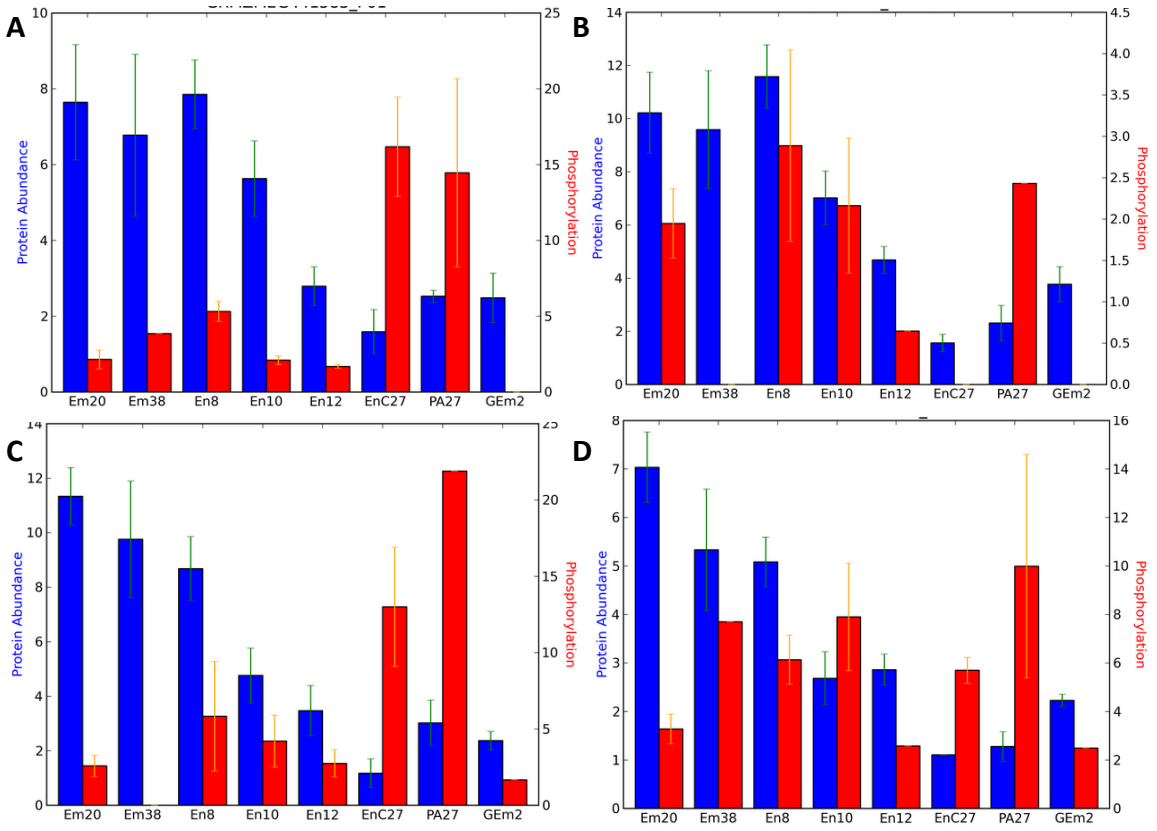

Figure 3.19 Normalized abundance of total and phosphorylated maize AGO1 proteins from maize embryo, endosperm and aleurone of different stages.

These four graphs were generated by Atlas of Maize Proteotypes (Walley, in preparation; http://maizeproteome.ucsd.edu/). Blue bars in each graph represent the normalized levels of total AGO1 protein and red bars the normalized level of phosphorylated AGO1 from each sample (as indicated in the X axis).
A. Zma-AGO1a; B. Zma-AGO1b; C. Zma-AGO1c; D. Zma-AGO1e

Em20: 20 DAP embryo; Em38: 38 DAP embryo; En8: 8 DAP endosperm; En10: 10 DAP endosperm; En12: 12 DAP endosperm; EnC 27: Upper part of 27 DAP endosperm; PA27: 27 DAP pericarp + aleurone; Gem2: 2 DAI embryo

DAP: Days After Pollination; DAI: Days After Imbibition

Modes of miRNA action might differ in a tissue- and temporal- specific manner

Based on our data and previous findings, one can easily argue that maybe miRNA regulatory mechanisms alternate in a tissue-/temporal-specific manner. Indeed, evidence has suggested that miRNAs might behave differently in various tissue types. For instance, miRNA159 has been known to regulate members of the MYB transcription family in Arabidopsis. Studies have shown that in vegetative tissues, the transcript levels of MYB33 and MYB65 become negligible due to miR159-directed cleavages (Reyes and Chua, 2007; Alonso-Peral et al., 2010; Alonso-Peral et al., 2012). However, recent evidence suggested that in germinating Arabidopsis seeds, especially at early stages of germination, even though their expression patterns co-localizes, the synthesis of the MYB33 protein was affected by miR159 to a lesser extent (Alonso-Peral et al., 2012). This observation can be explained by the difference in miR159 action modes. On the one hand, in vegetative tissue, miR159-directed transcript cleavage reduces the MYB33 transcripts to a minimum level. On the other hand, in germinating seeds, miR159 does not predominantly act to cause the transcript cleavage but instead, represses the MYB33 translation to subtly modulate the MYB33 expression. This theory would explain the experimental observation that even when miR159 and MYB33 are co-expressed in aleurone and embryos, the MYB33 protein was still produced, although to a much less degree.

Sequence contexts vs. miRNA modes of operation

Palatnik and colleagues argued that the sequence requirement for a plant miRNA to act either as a transcript slicer or translational repressor should be similar in plants. He based this argument on the fact that no obvious translational effect was observed on miRNAs targets with various complementarities (Palatnik et al., 2007). A previous study on miR165/166 and their target *PHB* revealed that target mismatches between positions 3 and 11 from the 5' end of miR165/166 caused up to a 200-fold reduction of the miRNA-directed cleavage, while only minor reductions were caused by mismatches at the 3' end of the miRNA (Mallory et al., 2004b). They thus proposed a "two-step" model where the miRISC firstly presented the 5' end of miRNAs for nucleation ("core pairing"; step1), followed by annealing of the central region and 3' end of miRNAs to targets (step2; Mallory et al., 2004). In this scheme, in which the initial 5' core pairing was the rate-limiting step, nucleation for mismatched core pairs would be slower and less stable than those with perfect matches. Consequently, miRNA: target hybrids with higher complementarities within the "core pairing" region would exhibit higher cleavage rates due to better nucleation efficiencies.

This "two step" model might shed light on understanding our observation of a relatively high degree of polysomal association for miR319 in our studies. In Arabidopsis, miR319 targets several members from the TCP transcription factor family. It has been pointed out by several studies that miR319:TCP hybrids have the highest degree of mismatches among all the known miRNA:target duplexes (Palatnik et al., 2007; Nag et al., 2009). While miR319:TCP hybrids display perfect pairing within the region corresponding to positions 2 through 13 on

miR319, they all have substantial mismatches and G:U wobbles from position 14 to the extreme 3' end. Consequently, the nucleation may be less favorable for miR319:TCP than it is for other better-matched miRNA:target pairs. In our model, we propose that AGO1 acts as a ribosome-associated protein during the miRNA:target recognition process. Therefore, we reasoned that the ribosome could act as an anchor to permit a longer residence time of AGO1 on the target site, thus allowing better formation of miR319:target hybrids. In non-polysomal fractions, however, the miR319:target hybrid formation is presumably more difficult without an apparent anchor/platform to stabilize the base-paring process. Consequently, this potential difference in the miR319:target nucleation efficiency may contribute to the observed enrichment of miR319 in polysomal fractions.

Modes of miRNA action from a functional perspective

In Arabidopsis, miR156 and miR172 have been shown to work antagonistically to regulate the juvenile-to-adult vegetative phase transition (Fornara and Coupland, 2009; Poethig, 2009; Wang et al., 2009b; Jung et al., 2011; Yang et al., 2011b). While miR156 targets 10 members of the SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) family of transcription factors, miR172 targets 6 APETALA2-LIKE (AP2-like) transcription factors (Wu et al., 2009). Constitutive expression of SPL proteins produces an early flowering phenotype and promotes the early production of trichomes on the abaxial surface of the leaf (Wu and Poethig, 2006; Gandikota et al., 2007). Conversely, targets of miR172 have an opposite role in regulating plant phase transition. Plants lacking the miR172 targets TARGET OF EAT1 and 2 (TOE1 and TOE2*)* exhibit early flowering, whereas plants overexpressing them and

other miR172 targets display a delay in flowering (Aukerman and Sakai, 2003; Jung et al., 2007).

As discussed earlier, modulation of gene expression by translational control is advantageous in biological contexts where transient or rapid responses are needed for physiological or metabolic adaptations in plants. Consistent with this idea, both miR156 and miR172 have been shown to act predominantly as translational repressors rather than causing transcript degradation (Chen, 2004; Gandikota et al., 2007).

Whether the miRNA-mediated translational repression occurs in a tissue-/temporal-, or a functionally dependent manner, the presence of protein effectors is required. And these effectors potentially need to be expressed in a coordinated manner for this mechanism to act in response to various developmental and environmental stimuli. So far in plants, only one of the characterized effectors of this translational control, AGO10, has been demonstrated to be expressed in a tissue-specific manner (Mallory et al., 2009; Zhu et al., 2011b). In contrast, other effectors including DCP1, DCP2, VSC, and KTN are universally expressed. Therefore, other unknown factors may be present that are involved in this regulatory pathway, and characterization of these factors will result in a more thorough understanding of this miRNA-mediated translational control mechanism.

Future Directions

As the core of the miRNA-mediated regulatory pathway, plant AGO1 proteins have two major roles in post-transcriptional control of gene expression. First, they direct the cleavage of target transcripts. Second, they also contribute to negatively regulate protein synthesis via a still unclear mechanism. Due to the predominance of miRNA-guided target cleavage events, it is difficult to mechanistically dissect or assess the biological significance of miRNA-mediated translational control in plants.

Based on our work and previous studies, we propose a model where the translational regulation by miRISCs is integrated into the translational machinery. In this model, we speculate that phosphorylation of AGO1 might be the key to program the miRISC complex into exerting translational control on target protein production. Moreover, we propose that AGO1 proteins become phosphorylated on polysomes via the activity of a PKC-like kinase, which is potentially recruited to polysomes by the ribosomal protein RACK1. To test this model, one could do the following experiments:

First of all, the most straightforward way to test the "phosphorylation theory" is to isolate the polysome-associated AGO1 proteins from the polysome-free AGO1 proteins and subject them to mass spectrometry analyses, respectively. If the model is true, it is expected that an enrichment of phosphorylated peptides (possibly with localized phosphorylated sites as shown in Figure 3.17) would be observed in the polysomal fraction of AGO1 proteins.

Second, to test if phosphorylation is essential for transforming AGO1 from a "slicer" into a translational repressor, one could make transgenic plants expressing FLAG-tagged AGO1 protein with a mutagenized phosphorylation site. By replacing the corresponding serine with an amino acid that cannot be phosphorylated, such as an alanine, in theory the role of AGO1 as the translational repressor will be abolished. Consequently, one should be able to observe in transgenic plants a lesser degree of FLAG-AGO1 proteins in polysomal fractions (even if the mutagenized FLAG-AGO1 still binds to polysomes, it will direct transcript cleavages rather than translational inhibition. Therefore, the miRISC:target ribonucleoprotein complexes will quickly dissociate from polysomes as the cleavage occurs.) Conversely, by replacing the phosphorylation site with a glutamate, which mimics the structure and negative charge status of a phosphorylated serine, we should be able to create a "dominant negative" AGO1 that potentially functions only as a translational repressor, without any slicing activity. Notably, if phosphorylation of AGO1 indeed is the factor that programs miRISCs into functioning as translational repressors, transgenic plants expressing this "dominant negative" AGO1 (in an *ago1* background) proteins will be a valuable system to mechanistically dissect the biological significance of miRNA-mediated translational control from the transcript cleavages.

Materials and Methods

Isolation of non-polysomal (NP) and polysomal RNAs (PS)

Maize (*Zea mays*) inbred line B73 was used in this study. The procedure used to isolate ribosome-associating RNAs was essentially the same as described previously (Zanetti et al., 2005; Mustroph et al., 2009). In summary, dissected maize endosperm was homogenized in Polysome Extraction Buffer (PEB) at a ratio of 1:2 (w/v), using a POLYTRON homogenizer. The homogenized tissue was incubated on ice for 10 min and subjected to centrifugation at 16,000xg at 4 ℃ for 15 min. The supernatant was filtered through 4 layers of Miracloth (Andwin Scientific, Schaumburg, IL, USA) to remove all the remaining cell debris and then loaded onto a 2M sucrose pad and subjected to centrifugation for 3 hrs at 46,100rpm (Beckman SW50.1 rotor) at 4℃. After the ultracentrifugation step, the upper phase was used for RNA extraction to yield non-ribosomal RNAs, while the pellet was re-suspended in Resuspension Buffer (RB). Resuspended polysomes were then separated through a 15–60% (v/v) exponential sucrose density gradient at 37,500 rpm by ultracentrifugation (4°C, Sorvall TST41, 150 min). 12 to 14 fractions of approximately 10ml were obtained by use of a gradient fractionator connected to an UA-5 detector (Bio-Rad, Hercules, CA, USA). Fractions 1–3 (sucrose gradient region containing the ribosomal subunits and 80S monosomes) and fractions 4-6, 7-8, 9-11, and 12-14 (gradient region containing disomes and complexes of greater density) were combined, respectively (PS: F1 through F5). The pooled gradient fractions were mixed with an equal volume of 8 m guanidine hydrochloride and vortexed for 3 min. RNA was precipitated and collected by addition of 1.5 volume of ethanol, storing overnight at −20°C, and subjected to centrifugation at 12, 100 x *g* for 45 min

at 4℃. The RNA pellet was re-suspended in 450 µl of RNase-free water, and the RNA was recovered by using TRizol LS according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA).

Plants and plant growth conditions

The maize (*Zea mays*) inbred B73 (+) was grown and self-pollinated at the Central Crops Research Station, Clayton, North Carolina, during summer field seasons. Kernels from well-filled ears were harvested 10, 18 and 27 d after pollination between 7:30 and 9:30 AM, immediately frozen in liquid nitrogen, and stored at −80°C until use.

Arabidopsis seeds were stratified at 4℃ for 2 days before plating on ½ MS plates. Transgenic *35S-HF-RPL18* and Col-0 plants were grown at 25℃ under16hr/8hr light conditions for 24hr or 20 days before being harvested for RNA extraction and immunoprecipitations.

Immunoprecipitation, protein and RNA extraction

Immunoprecipitation using anti-AGO1 antibodies (Agrisera, Vännäs, Sweden) was carried out as described previously (Mi et al., 2008) with some modifications. In brief, seeds were frozen in liquid nitrogen and ground in polysome extraction buffer. To separate ribosome-associated RNAs from ribosome-free fractions, the same protocol used to separate maize ribosomal fractions was used here. After the ultracentrifugation, 4ml of supernatant containing the ribosome-free RNAs was obtained. The ribosomal pellet was suspended in

4ml of PEB buffer. Then anti-AGO1 antibody was added to both ribosomal and non-ribosomal fractions at a ratio of 1:500 (v/v), while in the negative control this step was skipped. For subsequent steps, a standard immunoprecipitation protocol was followed (Mi et al., 2008). The αAGO1-immunoprecipitates were pulled down by protein A agarose beads following the manufacturer's protocol (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoprecipitation of polysomes using the anti-FLAG immobilized beads (Sigma-Aldrich, MO, USA) was carried out as previously described (Zanetti et al., 2005; Mustroph et al., 2009).

RNAs and proteins from the immunoprecipitates were extracted by TRizol LS (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. For immuno-dot-blotting, a goat-anti-rabbit secondary antibody conjugated with an alkaline phosphatase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used and the subsequent chromogenic detection was carried out using the 1-Step NBT/BCIP system (Thermo Scientific, Rockford, IL, USA).

Northern Blot

The protocol for the Northern blot used in this study was the same as described previously (Kim et al., 2010). Essentially, fractionated polysomes from sucrose fractions extracted and separated by Urea-PAGE (15%) prior to transfer, crosslinking, and probing with DIG-labeled LNA probes (miR167, Exiqon, Woburn, MA, USA).

miRNA RT-qPCR and primers

MiRNA RT-qPCR was performed as previously described (Chen et al., 2005). In short, prior to RNA extraction, the total volumes of ribosomal/polysomal and non-ribosomal/non-polysomal fractions were spiked with 0.1ng synthetic human let-7 miRNA as the internal/loading control for the RT-qPCR and semi-quantitative RT-PCR studies. Approximately 600ng of total RNA was used for each miRNA reverse transcription (RT) reaction. Each RT reaction was normally multiplexed using 4 to 6 different miRNA-specific stem-loop RT primers at concentrations of 50nM, respectively. The corresponding cDNAs were diluted 10-fold and subjected to RT-qPCR assays using PerfeCTa® SYBR® Green SuperMix, Low ROX™ (Quanta BioSciences, Gaithersburg, MD, USA) on a Stratagene Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA, USA). Results obtained from 3 biological replicates were analyzed; averaged values were taken with error bars representing the standard deviation (For polysome profiles for each individual replicate, refer to Figure S1 to Figure S4). Primers used in the miRNA RT-qPCR and semi-quantitative RT-PCR assays were listed as follows:

18S ath- rRNA
Forward primer 5' AGTCATCAGCTCGCGTTGACT 3'
Reverse primer 5' CTTCACCGGATCATTCAATCG 3'

Ath-U2
Forward primer 5' CACAGAGCAGAAAAAGTTCCTCCGT 3'
Reverse primer 5' TGTTCTCTGTGTTCGTTGCCACTG 3'

Universal Reverse Primer: 5' GTGCAGGGTCCGAGGT 3' (for miRNA qRT-PCR)

Ath(zma)-miR173 RT-primer
5' GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGATT 3'

Ath-miR173-F 5’ GATCttcgcttgcagagaga 3’

Ath(zma) -miR390 RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCGC 3’
Ath-mi390-F 5’ GTGCAGGGTCCGAGGT 3’

Ath(zma)-miR156 RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC 3’
Ath-miR156-F 5’ GGCCCtgacagaagagagt 3’

Ath(zma)-miR159 RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGTGC 3’
Ath-miR159-F 5’ GCCGCtttggattgaaggga 3’

Ath-miR165/166 RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGGGA 3’
Ath-miR165/166-F 5’ GCTAtcggaccaggctcat 3’

Ath(zma)-miR167ab RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGATC 3’

Ath(zma)-miR167cd RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCAGAT 3’
Ath-miR167-F 5’ GCATCtgaagctgccagcat 3’

Ath-miR168 RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCCCG 3’
Ath-miR168-F 5’ GTACtcgcttggtgcaggt 3’

Ath(zma)-miR169 RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGGCA 3’
Ath-miR169-F 5’ GCCCtagccaaggatgact 3’

Ath(zma)-miR172 RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG 3’
Ath-miR172-F 5’ GCCGCggaatcttgatgatg 3’

Ath-miR319RT RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGAG 3’
Ath-miR319-F 5’ CGGTttggactgaagggag 3’

Ath-miR398 RT-primer
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGGGG 3’

ath-miR398-F 5’ GCGGtgtgttctcaggtca 3’

Zma-miR166 RT
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGGAA 3’
Zma-miR166-F 5’ GCAT tcggaccaggcttca 3’

Zma-miR319 RT
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGAGC 3’
Zma-miR319-F 5’ CGGTttggactgaagggtg 3’

Zma-miR398 RT:
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGGGGG 3’
Zma-miR398-F 5’ GCGGtgtgttctcaggtcg 3’

Hsa-Let7
5’ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCACA 3’
Hsa-Let7-F 5’ gcccTGAGGTAGTAGGTTG 3’

Zma-17S
Forward primer 5’ AGTCATCAGCTCGCGTTGACT 3’
Reverse primer 5’ CTTCACCGGATCCATTCAATCG 3’

Zma-ACT
Forward primer 5’ GACCGAGGCCCCTCTCA 3’
Reverse primer 5’ CATTCGAAGGTTTCAAACATAATCTG 3’

TAS1B
Forward primer 5’ AGGTTCTTAGATCAGGTTCCGC 3’
Reverse primer 5’ AGACCGGGACATCGAACCTA 3’

TAS1A
Forward primer 5’ GGTTCCGCCTTTAGATCGAGT 3’
Reverse primer 5’ CTCCTACATGGCTGGTGACA 3’

TAS2
Forward primer 5’ AGGTTGGGTTTGGGAGTGAG 3’
Reverse primer 5’ AAACAGAGAGGAGGAGCCGT 3’

TCP2
Forward primer 5’ ACCGGCAGAATCCAACGGCG 3’
Reverse primer 5’ AAGCCACCACCTGATGCGGC 3’

TCP3

Forward primer 5’ CCACCATCATCATCCCTACC 3’
Reverse primer 5’ CCTTGAAACCGTGCTGGTAT 3’

TCP4
Forward primer 5’ ACGTCGTTTCAGCCAGTTCT 3’
Reverse primer 5’ TGGAGATGGATTGGTGATGA 3’

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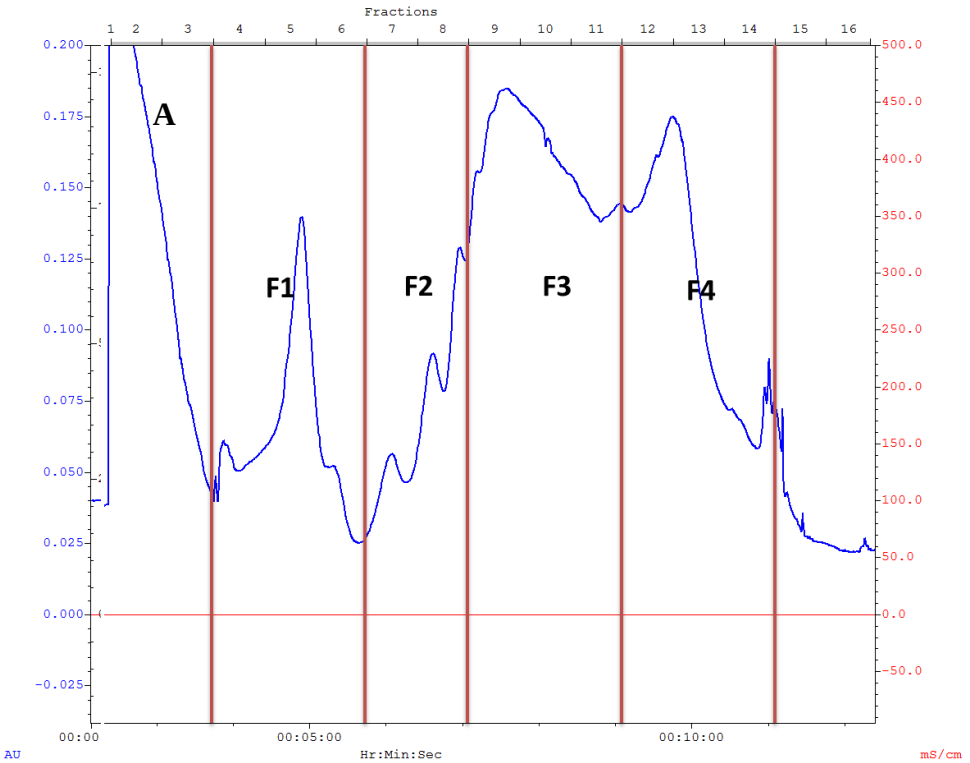
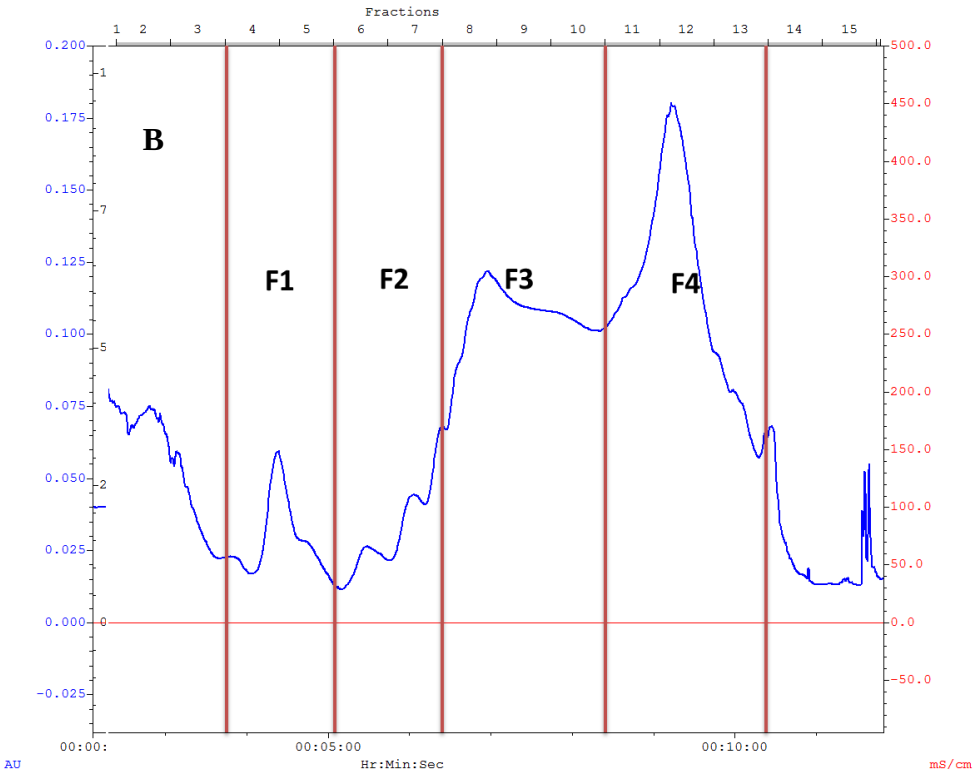
Supplemental Data

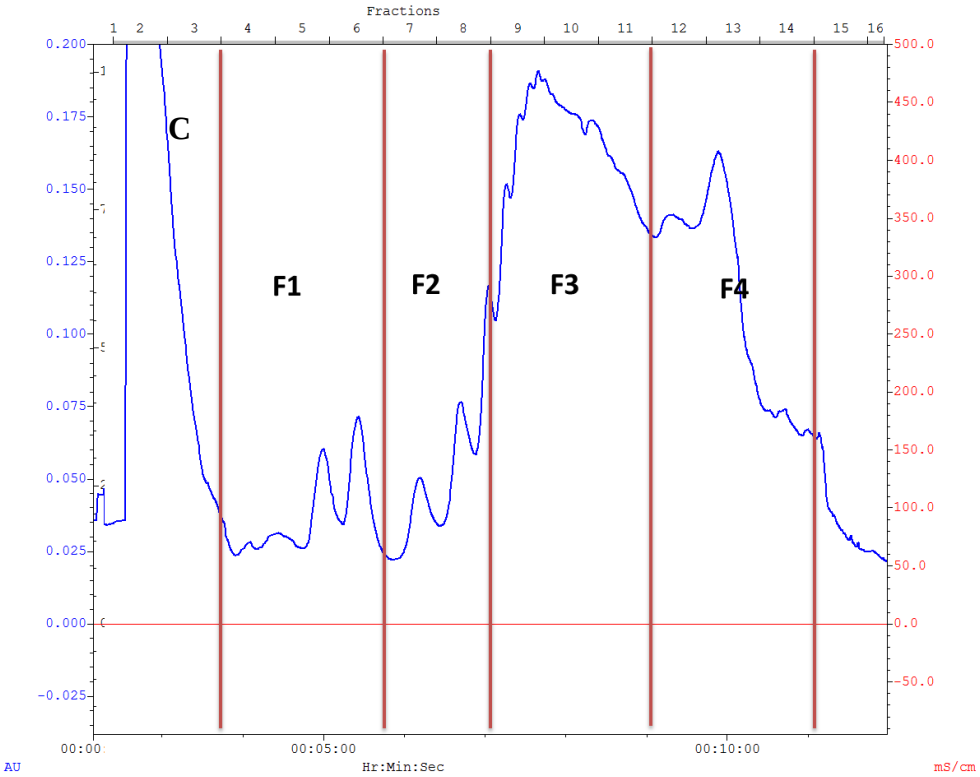
Figure S1. Polysome profiles of 10 DAP endosperm samples.

Polysomes were fractionated through sucrose gradients by differential ultracentifugation. RNAs were recovered using Trizol-LS. F1- F4: Fractionated polysomes with increasing sizes.

A. Biological replicate 1; B. Biological replicate 2; C. Biological replicate 3


Fractions
A
F1
F2
F3
F4
AU
Hr:Min:Sec
mS/cm


Fractions
B
F1
F2
F3
F4
AU
Hr:Min:Sec
mS/cm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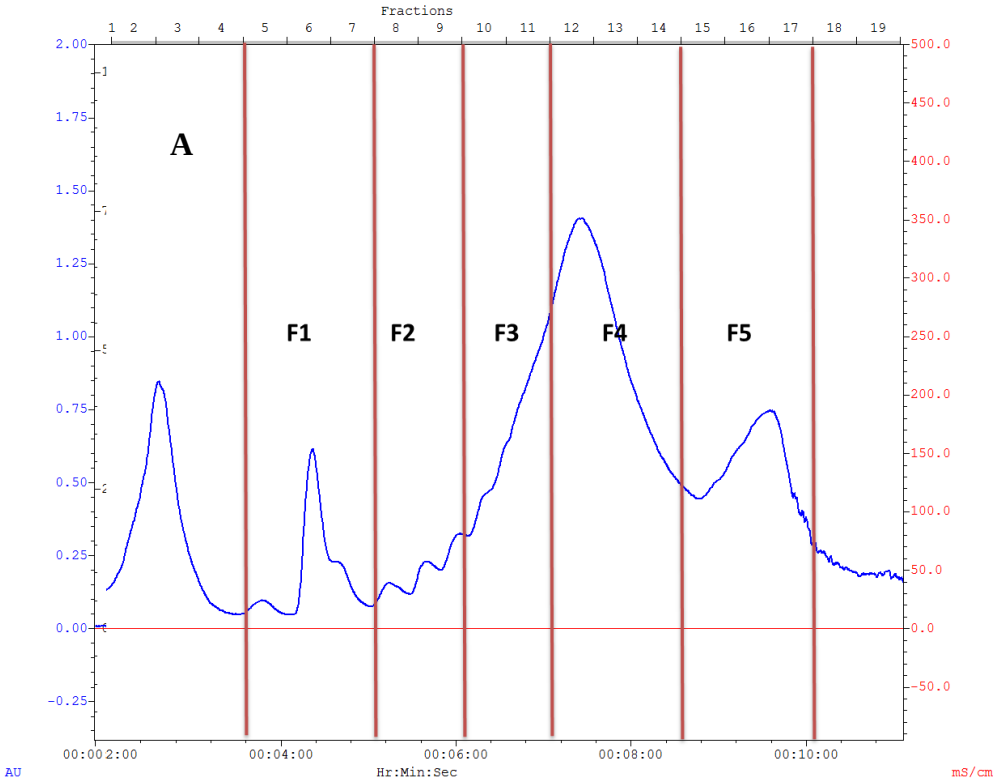
C
F1
F2
F3
F4
0.200
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
-0.025
500.0
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
-50.0
00:00
00:05:00
00:10:00
Hr:Min:Sec
AU
mS/cm

Figure S2. Polysome profiles of 18 DAP endosperm samples.

Sample was prepared the same way as described in Figure S1. F1- F5: Fractionated polysomes with increasing sizes

A. Biological replicate 1; B. Biological replicate 2; C. Biological replicate 3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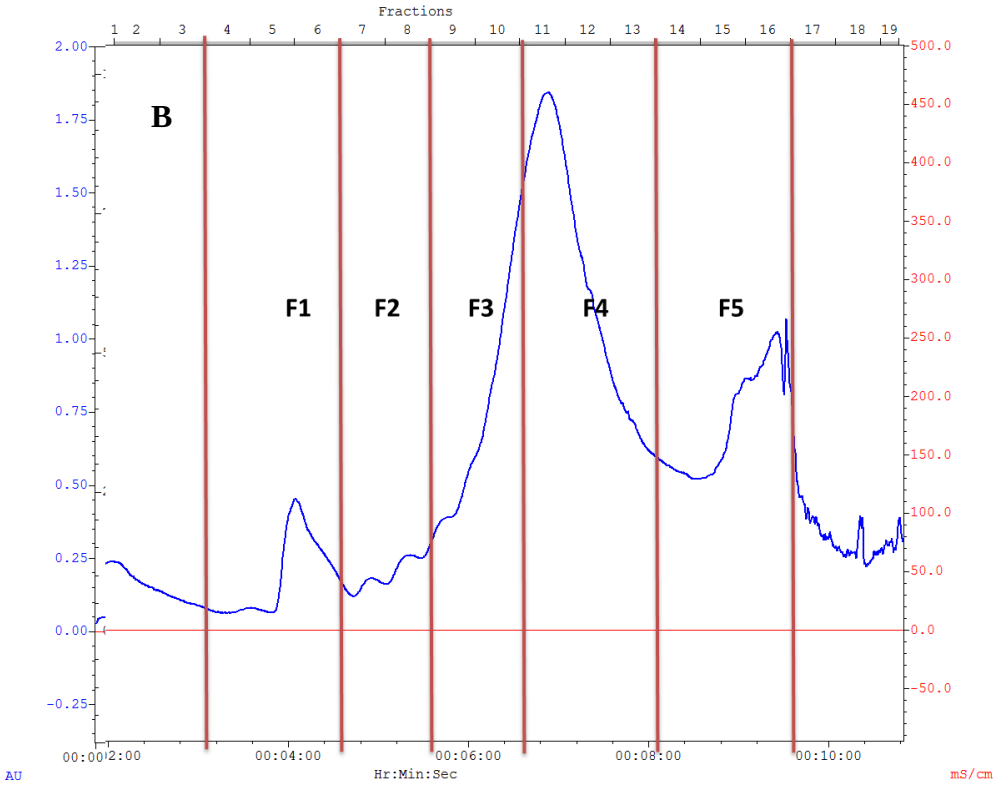
A
F1
F2
F3
F4
F5
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
500.0
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
-50.0
00:00
2:00
00:04:00
00:06:00
00:08:00
00:10:00
AU
Hr:Min:Sec
mS/cm


Fractions
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B
F1
F2
F3
F4
F5
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
-0.25
500.0
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
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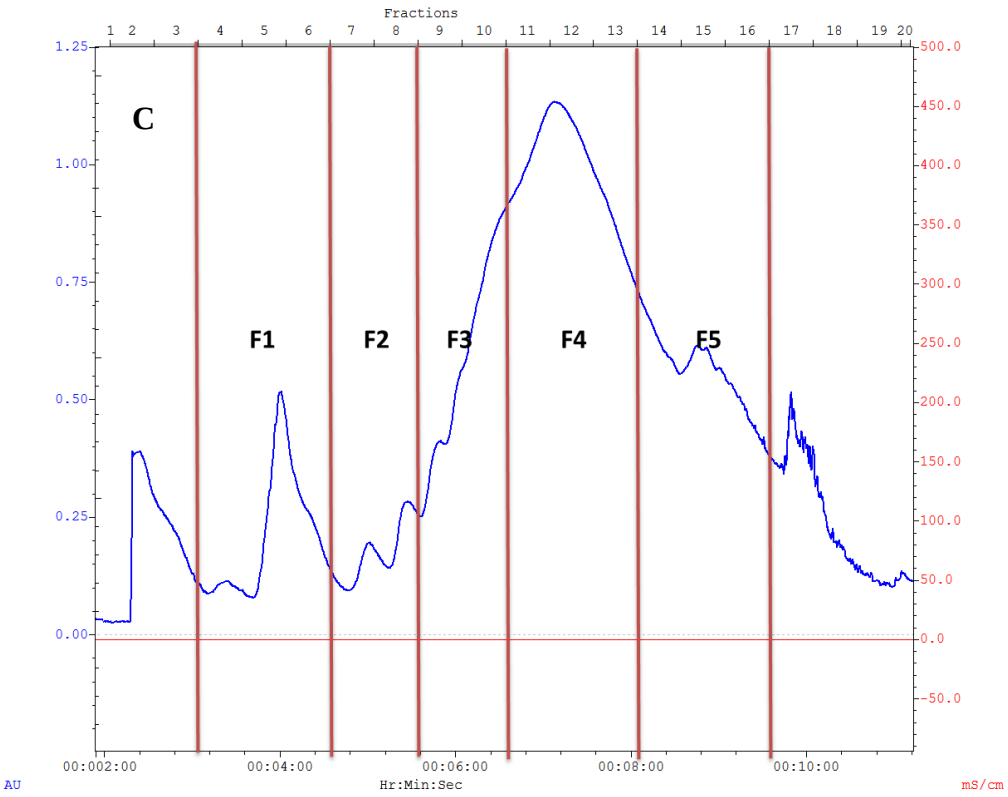
Hr:Min:Sec
mS/cm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C
F1
F2
F3
F4
F5
1.25
1.00
0.75
0.50
0.25
0.00
500.0
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
-50.0
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00:06:00
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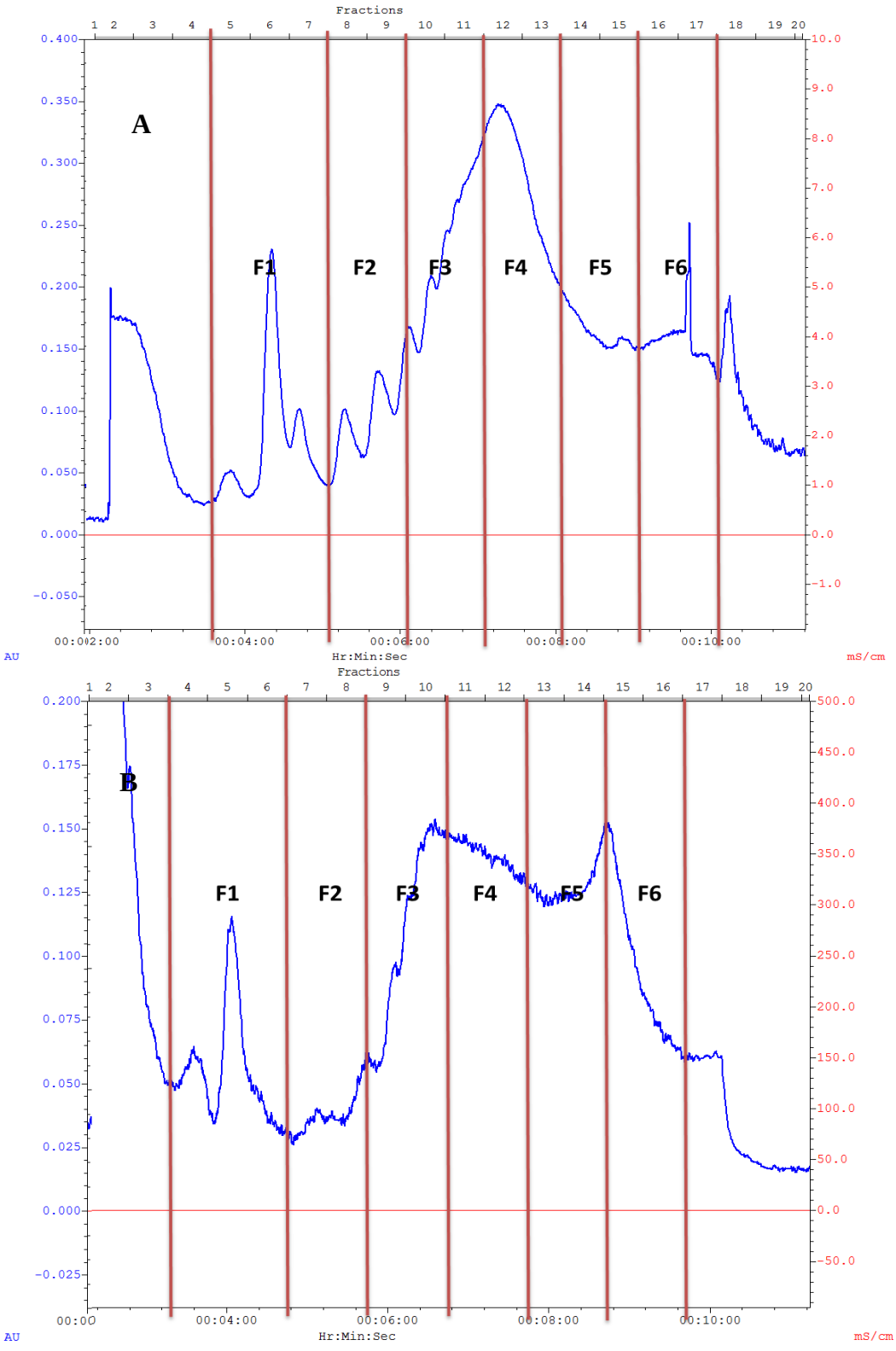
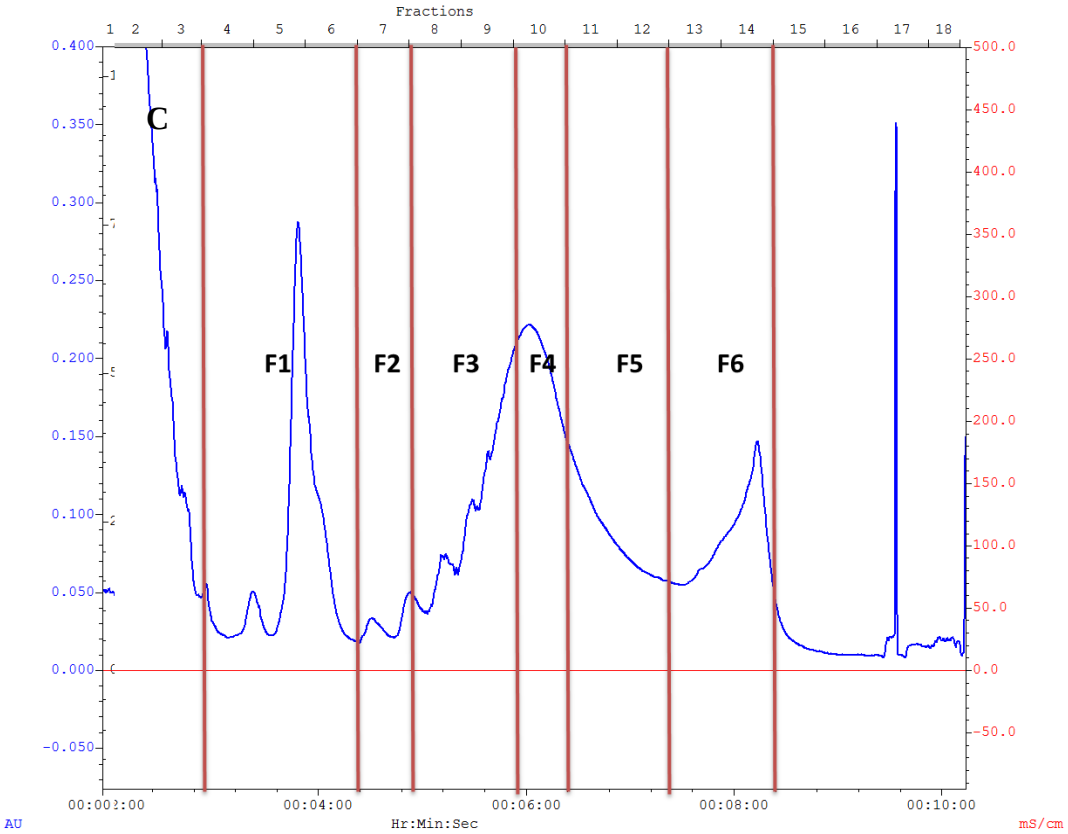
00:10:00
Hr:Min:Sec
AU
mS/cm

Figure S3. Polysome profiles of 18 DAP embryo samples.

Sample was prepared the same way as described in Figure S1. F1- F6: Fractionated polysomes with increasing sizes

A. Biological replicate 1; B. Biological replicate 2; C. Biological replicate 3


Fractions
A
F1
F2
F3
F4
F5
F6
AU
Hr:Min:Sec
mS/cm
B
F1
F2
F3
F4
F5
F6
AU
Hr:Min:Sec
mS/cm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C
F1
F2
F3
F4
F5
F6
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0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
-0.050
500.0
450.0
400.0
350.0
300.0
250.0
200.0
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0.0
-50.0
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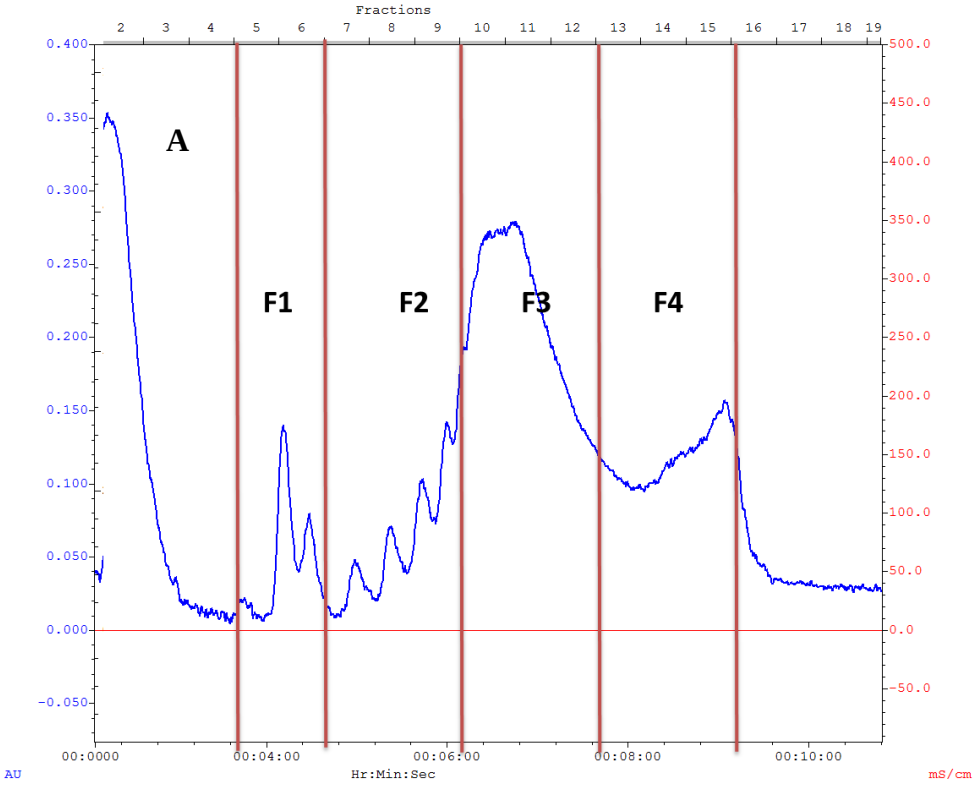
00:08:00
00:10:00
AU
Hr:Min:Sec
mS/cm

Figure S4. Polysome profiles of 27DAP embryo samples.

Sample was prepared the same way as described in Figure S1. F1- F4: Fractionated polysomes with increasing sizes

A. Biological replicate 1; B. Biological replicate 2; C. Biological replicate 3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F2
F3
F4
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500.0 450.0 400.0 350.0 300.0 250.0 200.0 150.0 100.0 50.0 0.0 -50.0
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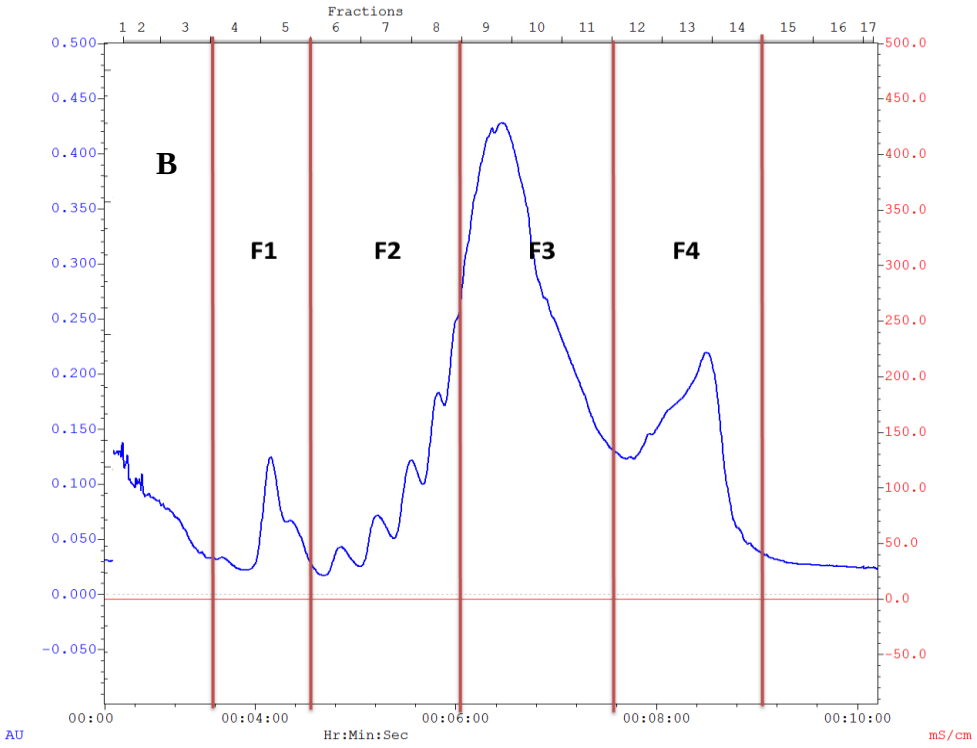
Hr:Min:Sec
AU
mS/cm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F2
F3
F4
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500.0 450.0 400.0 350.0 300.0 250.0 200.0 150.0 100.0 50.0 0.0 -50.0
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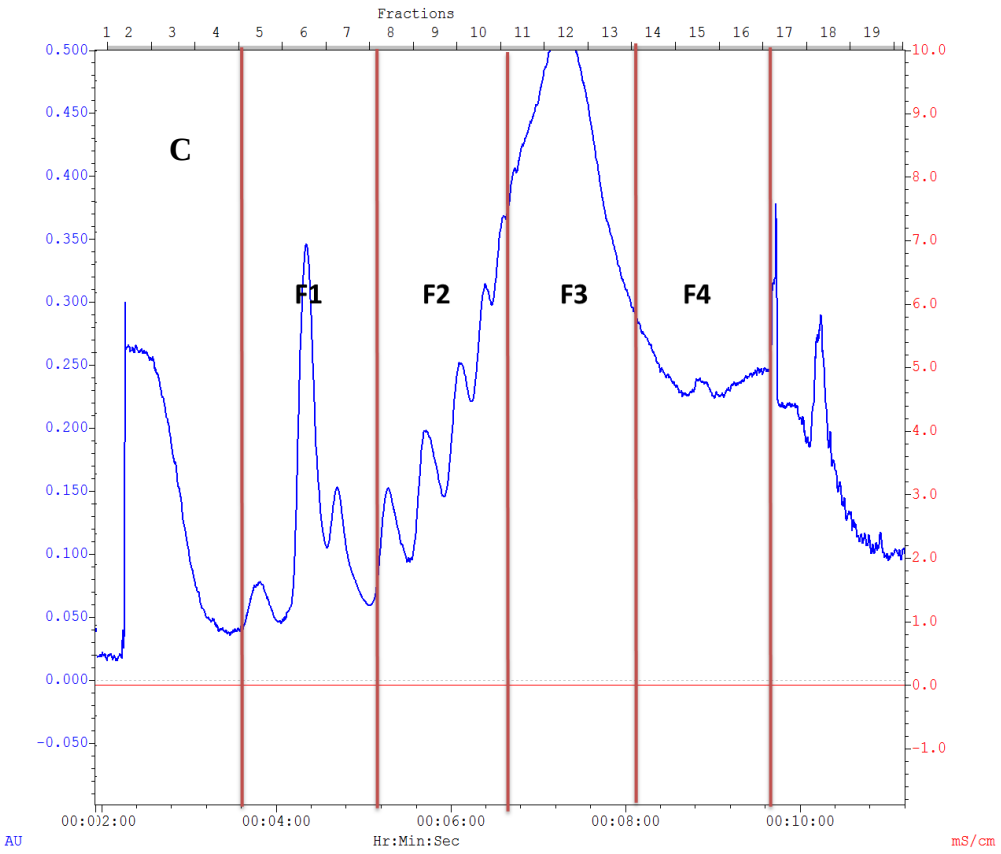
Hr:Min:Sec
AU
mS/cm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F1
F2
F3
F4
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
-0.050
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
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Hr:Min:Sec
AU
mS/cm

Figure S5. Surface accessibility and secondary structure prediction of the Arabidopsis AGO1 protein (only PIWI domain is shown here; Petersen et al., 2009).

Column 1: Class assignment - B for buried or E for Exposed

Column 2: Amino acid; Column 3: Sequence name; Column 4: Amino acid number

Column 5: Relative Surface Accessibility – RSA; Column 6: Absolute Surface Accessibility; Column

7: Z-fit score for RSA prediction;

Column 8: Probability for Alpha-Helix; Column 9: Probability for Beta-strand; Column 10:

Probability for Coil

Yellow highlighted: "DHH" catalytic amino acid residues.

Green highlighted: the S1001 phosphorylation site.

PIWI domain

B I	gi_11386626_sp_O04379.1_AGO1_ARATH	678	0.013	2.442	1.602	0.000	0.983	0.017
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	679	0.017	2.613	1.334	0.001	0.959	0.040
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	680	0.017	3.127	1.204	0.001	0.959	0.040
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	681	0.036	6.647	0.741	0.003	0.718	0.279
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	682	0.133	18.887	-0.104	0.005	0.336	0.660
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	683	0.503	72.540	-0.430	0.005	0.015	0.979
E N	gi_11386626_sp_O04379.1_AGO1_ARATH	684	0.581	85.102	-1.504	0.016	0.005	0.979
E N	gi_11386626_sp_O04379.1_AGO1_ARATH	685	0.488	71.385	-1.302	0.016	0.005	0.979
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	686	0.382	30.048	-0.469	0.502	0.002	0.495
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	687	0.646	75.676	0.372	0.782	0.003	0.216
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	688	0.145	26.604	1.020	0.923	0.002	0.076
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	689	0.051	10.941	0.842	0.923	0.002	0.076
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	690	0.398	31.354	0.514	0.938	0.007	0.055
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	691	0.399	57.453	0.649	0.938	0.007	0.055
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	692	0.049	8.899	0.495	0.938	0.007	0.055
B K	gi_11386626_sp_O04379.1_AGO1_ARATH	693	0.061	12.589	0.637	0.879	0.010	0.111
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	694	0.424	97.119	1.135	0.879	0.010	0.111
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	695	0.149	27.639	0.448	0.802	0.014	0.185
B C	gi_11386626_sp_O04379.1_AGO1_ARATH	696	0.048	6.781	-1.127	0.751	0.050	0.199
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	697	0.326	57.040	-1.580	0.455	0.046	0.498
E T	gi_11386626_sp_O04379.1_AGO1_ARATH	698	0.334	46.256	-0.502	0.307	0.165	0.527
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	699	0.513	89.691	-0.583	0.216	0.235	0.548
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	700	0.268	48.998	0.370	0.056	0.142	0.802
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	701	0.341	26.852	-1.350	0.018	0.047	0.935
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	702	0.079	14.578	0.105	0.019	0.141	0.840
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	703	0.147	22.625	0.277	0.021	0.451	0.528
B S	gi_11386626_sp_O04379.1_AGO1_ARATH	704	0.072	8.450	-0.607	0.023	0.655	0.322
B Q	gi_11386626_sp_O04379.1_AGO1_ARATH	705	0.082	14.645	-0.235	0.021	0.756	0.223
B C	gi_11386626_sp_O04379.1_AGO1_ARATH	706	0.053	7.455	-0.766	0.085	0.775	0.140
B C	gi_11386626_sp_O04379.1_AGO1_ARATH	707	0.035	4.872	0.627	0.085	0.775	0.140
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	708	0.120	22.027	0.149	0.079	0.592	0.329
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	709	0.121	16.797	-0.248	0.453	0.248	0.299
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	710	0.484	99.477	0.673	0.605	0.105	0.290
E H	gi_11386626_sp_O04379.1_AGO1_ARATH	711	0.272	49.531	0.380	0.701	0.107	0.192
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	712	0.046	7.055	-1.515	0.561	0.047	0.393
E F	gi_11386626_sp_O04379.1_AGO1_ARATH	713	0.480	96.396	0.466	0.455	0.046	0.498
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	714	0.548	112.682	0.160	0.257	0.016	0.727
E M	gi_11386626_sp_O04379.1_AGO1_ARATH	715	0.453	90.585	-1.128	0.176	0.004	0.820
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	716	0.439	51.498	-1.027	0.176	0.004	0.820
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	717	0.515	105.874	-0.458	0.782	0.003	0.216
E Q	gi_11386626_sp_O04379.1_AGO1_ARATH	718	0.370	66.064	0.050	0.879	0.010	0.111
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	719	0.110	23.486	0.800	0.938	0.007	0.055
B M	gi_11386626_sp_O04379.1_AGO1_ARATH	720	0.047	9.385	0.074	0.970	0.001	0.030
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	721	0.172	18.910	-0.542	0.970	0.001	0.030
B N	gi_11386626_sp_O04379.1_AGO1_ARATH	722	0.118	17.290	-0.082	0.988	0.000	0.012
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	723	0.023	3.581	0.643	0.988	0.000	0.012
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	724	0.023	2.513	0.440	0.988	0.000	0.012
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	725	0.150	27.520	0.195	0.988	0.000	0.012
B K	gi_11386626_sp_O04379.1_AGO1_ARATH	726	0.150	30.773	0.987	0.975	0.003	0.022
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	727	0.019	3.607	1.030	0.975	0.003	0.022
B N	gi_11386626_sp_O04379.1_AGO1_ARATH	728	0.066	9.604	0.129	0.938	0.007	0.055
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	729	0.169	25.929	-0.808	0.782	0.003	0.216
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	730	0.271	55.765	-0.468	0.717	0.014	0.269
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	731	0.116	17.891	-0.645	0.339	0.016	0.645
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	732	0.441	34.683	-1.716	0.016	0.005	0.979
B G	gi_11386626_sp_O04379.1_AGO1_ARATH	733	0.244	19.242	-2.413	0.005	0.015	0.979
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	734	0.274	62.723	-0.430	0.021	0.279	0.699
B N	gi_11386626_sp_O04379.1_AGO1_ARATH	735	0.155	22.619	-1.354	0.004	0.420	0.576
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	736	0.135	18.766	0.368	0.004	0.616	0.381
E V	gi_11386626_sp_O04379.1_AGO1_ARATH	737	0.290	44.604	0.409	0.003	0.718	0.279
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	738	0.060	10.986	0.295	0.004	0.616	0.381
E V	gi_11386626_sp_O04379.1_AGO1_ARATH	739	0.355	54.594	-0.416	0.022	0.359	0.619
B D	gi_11386626_sp_O04379.1_AGO1_ARATH	740	0.297	42.827	-0.640	0.113	0.043	0.844
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	741	0.528	58.230	-1.475	0.339	0.016	0.645
E L	gi_11386626_sp_O04379.1_AGO1_ARATH	742	0.395	72.270	-1.458	0.430	0.016	0.555
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	743	0.461	53.982	-1.439	0.339	0.016	0.645
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	744	0.532	121.714	-1.358	0.257	0.016	0.727
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	745	0.568	130.118	-1.372	0.181	0.016	0.803
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	746	0.260	48.063	-0.489	0.058	0.017	0.925
E P	gi_11386626_sp_O04379.1_AGO1_ARATH	747	0.427	60.577	-1.192	0.053	0.043	0.903
E L	gi_11386626_sp_O04379.1_AGO1_ARATH	748	0.426	77.927	-1.560	0.053	0.043	0.903

B V	gi_11386626_sp_O04379.1_AGO1_ARATH	749	0.205	31.524	-2.105	0.053	0.043	0.903
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	750	0.339	39.684	-1.696	0.018	0.019	0.964
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	751	0.694	99.948	-1.363	0.016	0.005	0.979
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	752	0.340	77.952	-0.057	0.003	0.003	0.994
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	753	0.135	19.142	-0.145	0.005	0.015	0.979
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	754	0.037	5.090	-0.117	0.004	0.616	0.381
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	755	0.017	3.090	1.424	0.001	0.900	0.099
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	756	0.015	2.794	1.389	0.001	0.959	0.040
B F	gi_11386626_sp_O04379.1_AGO1_ARATH	757	0.014	2.750	1.625	0.001	0.959	0.040
B G	gi_11386626_sp_O04379.1_AGO1_ARATH	758	0.017	1.314	0.517	0.001	0.959	0.040
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	759	0.019	2.127	0.960	0.001	0.900	0.099
B D	gi_11386626_sp_O04379.1_AGO1_ARATH	760	0.033	4.726	-0.889	0.002	0.816	0.182
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	761	0.017	2.674	1.403	0.022	0.552	0.426
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	762	0.050	6.935	-0.649	0.004	0.420	0.576
B H	gi_11386626_sp_O04379.1_AGO1_ARATH	763	0.069	12.515	0.340	0.005	0.336	0.660
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	764	0.196	27.869	-0.372	0.004	0.085	0.910
E H	gi_11386626_sp_O04379.1_AGO1_ARATH	765	0.266	48.313	-1.017	0.018	0.019	0.964
E P	gi_11386626_sp_O04379.1_AGO1_ARATH	766	0.494	70.155	-2.121	0.016	0.005	0.979
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	767	0.524	41.270	-1.962	0.016	0.005	0.979
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	768	0.600	104.785	-2.398	0.018	0.019	0.964
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	769	0.594	85.610	-2.338	0.018	0.019	0.964
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	770	0.540	63.335	-1.507	0.005	0.045	0.951
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	771	0.373	43.762	-0.437	0.004	0.085	0.910
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	772	0.123	17.482	-0.421	0.004	0.197	0.799
B S	gi_11386626_sp_O04379.1_AGO1_ARATH	773	0.042	4.899	-2.351	0.004	0.616	0.381
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	774	0.045	8.380	0.017	0.001	0.900	0.099
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	775	0.016	1.818	0.903	0.001	0.959	0.040
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	776	0.045	4.904	-0.195	0.001	0.959	0.040
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	777	0.029	4.457	-0.477	0.001	0.959	0.040
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	778	0.043	6.609	-0.790	0.001	0.900	0.099
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	779	0.025	2.722	-0.039	0.003	0.718	0.279
B S	gi_11386626_sp_O04379.1_AGO1_ARATH	780	0.044	5.204	-3.092	0.004	0.616	0.381
B Q	gi_11386626_sp_O04379.1_AGO1_ARATH	781	0.147	26.290	-0.759	0.005	0.262	0.733
B D	gi_11386626_sp_O04379.1_AGO1_ARATH	782	0.187	27.004	-2.057	0.004	0.085	0.910
B W	gi_11386626_sp_O04379.1_AGO1_ARATH	783	0.283	68.013	-0.723	0.058	0.017	0.925
E P	gi_11386626_sp_O04379.1_AGO1_ARATH	784	0.640	90.788	-2.022	0.053	0.005	0.942
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	785	0.458	79.960	-1.246	0.053	0.005	0.942
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	786	0.130	23.976	-0.864	0.018	0.019	0.964
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	787	0.195	27.060	-1.972	0.019	0.141	0.840
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	788	0.296	60.825	0.230	0.004	0.420	0.576
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	789	0.051	10.856	-0.213	0.004	0.616	0.381
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	790	0.099	10.899	-0.826	0.003	0.718	0.279
B G	gi_11386626_sp_O04379.1_AGO1_ARATH	791	0.035	2.770	-3.426	0.001	0.900	0.099
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	792	0.102	18.603	-0.752	0.001	0.959	0.040
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	793	0.037	5.687	-1.174	0.001	0.959	0.040
B C	gi_11386626_sp_O04379.1_AGO1_ARATH	794	0.157	22.085	-0.104	0.001	0.959	0.040
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	795	0.227	25.004	-1.112	0.001	0.900	0.099
B Q	gi_11386626_sp_O04379.1_AGO1_ARATH	796	0.210	37.577	-0.658	0.004	0.514	0.481
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	797	0.609	67.090	-0.438	0.004	0.085	0.910
E H	gi_11386626_sp_O04379.1_AGO1_ARATH	798	0.555	100.882	-1.080	0.018	0.019	0.964
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	799	0.491	112.531	-0.925	0.052	0.084	0.864
E Q	gi_11386626_sp_O04379.1_AGO1_ARATH	800	0.382	68.189	-0.231	0.135	0.317	0.548
B E	gi_11386626_sp_O04379.1_AGO1_ARATH	801	0.286	49.912	-0.645	0.159	0.516	0.325
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	802	0.166	30.321	-0.320	0.268	0.505	0.227
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	803	0.109	20.165	-0.420	0.321	0.252	0.427
E Q	gi_11386626_sp_O04379.1_AGO1_ARATH	804	0.535	95.551	-0.615	0.502	0.102	0.396
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	805	0.438	63.073	-1.492	0.561	0.047	0.393
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	806	0.115	21.111	-0.947	0.751	0.050	0.199
E F	gi_11386626_sp_O04379.1_AGO1_ARATH	807	0.320	64.164	-0.097	0.751	0.050	0.199
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	808	0.507	104.228	0.520	0.751	0.050	0.199
B E	gi_11386626_sp_O04379.1_AGO1_ARATH	809	0.241	42.173	-1.194	0.717	0.014	0.269
B W	gi_11386626_sp_O04379.1_AGO1_ARATH	810	0.149	35.810	-1.068	0.717	0.014	0.269
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	811	0.548	112.806	-0.407	0.622	0.015	0.363
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	812	0.451	65.047	-0.871	0.622	0.015	0.363
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	813	0.268	38.029	-1.455	0.622	0.015	0.363
E Q	gi_11386626_sp_O04379.1_AGO1_ARATH	814	0.349	62.403	-1.391	0.660	0.049	0.291
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	815	0.386	79.482	-0.503	0.751	0.050	0.199
B G	gi_11386626_sp_O04379.1_AGO1_ARATH	816	0.235	18.534	-1.459	0.717	0.014	0.269
E V	gi_11386626_sp_O04379.1_AGO1_ARATH	817	0.361	55.547	-0.564	0.802	0.014	0.185
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	818	0.239	36.750	-0.635	0.802	0.014	0.185
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	819	0.127	17.573	-0.716	0.879	0.010	0.111
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	820	0.354	27.852	-0.658	0.923	0.002	0.076
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	821	0.477	37.571	-0.379	0.923	0.002	0.076
B M	gi_11386626_sp_O04379.1_AGO1_ARATH	822	0.136	27.134	0.450	0.923	0.002	0.076
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	823	0.092	16.983	-0.236	0.970	0.001	0.030
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	824	0.307	63.170	0.519	0.970	0.001	0.030

E E	gi_11386626_sp_O04379.1_AGO1_ARATH	825	0.446	78.004	0.596	0.970	0.001	0.030
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	826	0.054	9.961	0.414	0.970	0.001	0.030
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	827	0.041	7.562	0.142	0.970	0.001	0.030
E I	gi_11386626_sp_O04379.1_AGO1_ARATH	828	0.409	75.721	1.241	0.970	0.001	0.030
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	829	0.331	36.476	0.886	0.970	0.001	0.030
B F	gi_11386626_sp_O04379.1_AGO1_ARATH	830	0.053	10.677	-0.050	0.970	0.001	0.030
B R	gi_11386626_sp_O04379.1_AGO1_ARATH	831	0.245	56.174	0.387	0.970	0.001	0.030
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	832	0.624	142.782	0.805	0.923	0.002	0.076
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	833	0.527	61.729	-0.204	0.782	0.003	0.216
E T	gi_11386626_sp_O04379.1_AGO1_ARATH	834	0.438	60.792	-1.410	0.406	0.004	0.590
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	835	0.704	55.421	-0.806	0.053	0.005	0.942
E H	gi_11386626_sp_O04379.1_AGO1_ARATH	836	0.395	71.778	-0.295	0.018	0.019	0.964
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	837	0.302	62.039	0.864	0.018	0.047	0.935
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	838	0.091	12.970	-0.596	0.004	0.085	0.910
E L	gi_11386626_sp_O04379.1_AGO1_ARATH	839	0.322	59.050	0.228	0.004	0.420	0.576
B R	gi_11386626_sp_O04379.1_AGO1_ARATH	840	0.157	35.976	0.853	0.001	0.900	0.099
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	841	0.019	3.478	1.410	0.001	0.959	0.040
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	842	0.027	5.013	1.119	0.001	0.959	0.040
B F	gi_11386626_sp_O04379.1_AGO1_ARATH	843	0.027	5.479	0.683	0.001	0.959	0.040
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	844	0.039	8.398	0.076	0.001	0.959	0.040
B R	gi_11386626_sp_O04379.1_AGO1_ARATH	845	0.086	19.625	0.294	0.002	0.816	0.182
B D	gi_11386626_sp_O04379.1_AGO1_ARATH	846	0.153	21.975	-0.752	0.004	0.420	0.576
B G	gi_11386626_sp_O04379.1_AGO1_ARATH	847	0.150	11.829	-2.018	0.004	0.138	0.858
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	848	0.087	13.387	-0.546	0.018	0.088	0.893
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	849	0.302	35.383	-0.616	0.018	0.019	0.964
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	850	0.461	80.589	-0.957	0.502	0.002	0.495
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	851	0.325	25.609	-2.056	0.600	0.003	0.397
B Q	gi_11386626_sp_O04379.1_AGO1_ARATH	852	0.129	23.022	-1.008	0.694	0.003	0.303
B F	gi_11386626_sp_O04379.1_AGO1_ARATH	853	0.094	18.846	0.136	0.858	0.002	0.139
E Y	gi_11386626_sp_O04379.1_AGO1_ARATH	854	0.407	86.955	0.721	0.923	0.002	0.076
E Q	gi_11386626_sp_O04379.1_AGO1_ARATH	855	0.451	80.620	1.235	0.923	0.002	0.076
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	856	0.032	4.995	0.052	0.970	0.001	0.030
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	857	0.079	14.410	0.931	0.970	0.001	0.030
E L	gi_11386626_sp_O04379.1_AGO1_ARATH	858	0.492	90.049	1.242	0.970	0.001	0.030
E Y	gi_11386626_sp_O04379.1_AGO1_ARATH	859	0.408	87.190	1.481	0.970	0.001	0.030
B E	gi_11386626_sp_O04379.1_AGO1_ARATH	860	0.041	7.163	-1.450	0.970	0.001	0.030
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	861	0.054	9.961	0.916	0.970	0.001	0.030
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	862	0.502	72.396	1.525	0.970	0.001	0.030
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	863	0.306	33.743	1.337	0.970	0.001	0.030
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	864	0.020	3.718	0.970	0.988	0.000	0.012
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	865	0.311	71.288	1.137	0.988	0.000	0.012
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	866	0.548	112.621	1.491	0.988	0.000	0.012
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	867	0.092	10.138	-0.269	0.970	0.001	0.030
B C	gi_11386626_sp_O04379.1_AGO1_ARATH	868	0.042	5.855	-0.130	0.970	0.001	0.030
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	869	0.566	62.351	1.406	0.923	0.002	0.076
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	870	0.606	71.023	0.609	0.858	0.002	0.139
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	871	0.179	32.775	-0.339	0.694	0.003	0.303
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	872	0.377	65.809	-0.842	0.246	0.004	0.750
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	873	0.730	80.391	-0.471	0.053	0.005	0.942
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	874	0.747	58.813	-1.910	0.016	0.005	0.979
E Y	gi_11386626_sp_O04379.1_AGO1_ARATH	875	0.306	65.307	-0.559	0.018	0.019	0.964
E Q	gi_11386626_sp_O04379.1_AGO1_ARATH	876	0.394	70.368	0.377	0.005	0.015	0.979
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	877	0.044	6.229	0.251	0.004	0.085	0.910
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	878	0.168	23.868	0.762	0.004	0.616	0.381
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	879	0.020	3.074	1.291	0.001	0.900	0.099
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	880	0.022	3.079	0.794	0.001	0.959	0.040
B F	gi_11386626_sp_O04379.1_AGO1_ARATH	881	0.016	3.291	1.657	0.001	0.959	0.040
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	882	0.017	2.613	1.460	0.001	0.959	0.040
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	883	0.021	3.243	1.174	0.001	0.959	0.040
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	884	0.022	3.428	0.569	0.001	0.959	0.040
B Q	gi_11386626_sp_O04379.1_AGO1_ARATH	885	0.143	25.558	0.546	0.002	0.816	0.182
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	886	0.250	51.384	-0.269	0.004	0.420	0.576
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	887	0.369	84.570	0.383	0.020	0.205	0.775
B H	gi_11386626_sp_O04379.1_AGO1_ARATH	888	0.147	26.739	-0.012	0.022	0.359	0.619
B H	gi_11386626_sp_O04379.1_AGO1_ARATH	889	0.173	31.414	-0.185	0.022	0.552	0.426
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	890	0.069	9.584	-0.087	0.021	0.756	0.223
B R	gi_11386626_sp_O04379.1_AGO1_ARATH	891	0.087	19.992	0.364	0.018	0.846	0.136
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	892	0.046	8.478	-0.313	0.018	0.846	0.136
B F	gi_11386626_sp_O04379.1_AGO1_ARATH	893	0.071	14.350	0.433	0.021	0.756	0.223
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	894	0.102	11.251	-1.002	0.004	0.514	0.481
E Q	gi_11386626_sp_O04379.1_AGO1_ARATH	895	0.304	54.294	-0.880	0.020	0.205	0.775
E N	gi_11386626_sp_O04379.1_AGO1_ARATH	896	0.481	70.462	-1.293	0.018	0.088	0.893
E H	gi_11386626_sp_O04379.1_AGO1_ARATH	897	0.565	102.737	-1.482	0.053	0.043	0.903
E N	gi_11386626_sp_O04379.1_AGO1_ARATH	898	0.585	85.585	-1.909	0.053	0.043	0.903
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	899	0.585	84.226	-1.811	0.113	0.087	0.800
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	900	0.473	108.386	-1.412	0.113	0.087	0.800

E H	gi_11386626_sp_O04379.1_AGO1_ARATH	901	0.435	79.054	-0.922	0.113	0.087	0.800
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	902	0.463	54.299	-1.144	0.113	0.087	0.800
E V	gi_11386626_sp_O04379.1_AGO1_ARATH	903	0.569	87.455	-1.404	0.113	0.043	0.844
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	904	0.458	65.940	-1.852	0.053	0.043	0.903
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	905	0.514	117.775	-0.487	0.052	0.084	0.864
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	906	0.543	63.640	-2.106	0.018	0.047	0.935
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	907	0.417	32.786	-0.478	0.005	0.045	0.951
B N	gi_11386626_sp_O04379.1_AGO1_ARATH	908	0.153	22.428	-1.034	0.004	0.085	0.910
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	909	0.129	23.828	0.722	0.004	0.085	0.910
E L	gi_11386626_sp_O04379.1_AGO1_ARATH	910	0.349	63.865	-0.030	0.004	0.085	0.910
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	911	0.193	27.401	-0.948	0.005	0.045	0.951
B G	gi_11386626_sp_O04379.1_AGO1_ARATH	912	0.098	7.728	-2.967	0.004	0.085	0.910
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	913	0.078	10.874	-0.355	0.004	0.616	0.381
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	914	0.026	4.058	0.144	0.001	0.900	0.099
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	915	0.050	7.639	0.444	0.018	0.846	0.136
B D	gi_11386626_sp_O04379.1_AGO1_ARATH	916	0.088	12.623	-0.667	0.022	0.552	0.426
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	917	0.302	35.441	-0.068	0.019	0.141	0.840
B K	gi_11386626_sp_O04379.1_AGO1_ARATH	918	0.253	52.022	-1.051	0.019	0.141	0.840
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	919	0.045	8.306	-0.227	0.022	0.359	0.619
B C	gi_11386626_sp_O04379.1_AGO1_ARATH	920	0.131	18.378	-1.026	0.021	0.451	0.528
B H	gi_11386626_sp_O04379.1_AGO1_ARATH	921	0.247	45.002	0.149	0.019	0.141	0.840
E P	gi_11386626_sp_O04379.1_AGO1_ARATH	922	0.341	48.445	-0.652	0.018	0.088	0.893
E T	gi_11386626_sp_O04379.1_AGO1_ARATH	923	0.468	64.842	-0.535	0.018	0.088	0.893
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	924	0.309	54.000	-0.181	0.019	0.141	0.840
B F	gi_11386626_sp_O04379.1_AGO1_ARATH	925	0.155	31.008	0.406	0.005	0.262	0.733
B D	gi_11386626_sp_O04379.1_AGO1_ARATH	926	0.058	8.387	-0.678	0.004	0.514	0.481
B F	gi_11386626_sp_O04379.1_AGO1_ARATH	927	0.022	4.415	1.075	0.011	0.918	0.071
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	928	0.024	5.150	0.979	0.006	0.962	0.032
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	929	0.017	3.076	1.535	0.006	0.962	0.032
B C	gi_11386626_sp_O04379.1_AGO1_ARATH	930	0.026	3.580	0.877	0.011	0.918	0.071
B S	gi_11386626_sp_O04379.1_AGO1_ARATH	931	0.020	2.309	0.406	0.021	0.756	0.223
B H	gi_11386626_sp_O04379.1_AGO1_ARATH	932	0.067	12.151	-0.232	0.022	0.359	0.619
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	933	0.192	21.125	-0.099	0.064	0.216	0.721
B G	gi_11386626_sp_O04379.1_AGO1_ARATH	934	0.073	5.761	-0.780	0.056	0.142	0.802
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	935	0.205	37.869	-0.841	0.056	0.142	0.802
E Q	gi_11386626_sp_O04379.1_AGO1_ARATH	936	0.277	49.454	-0.136	0.056	0.142	0.802
B G	gi_11386626_sp_O04379.1_AGO1_ARATH	937	0.168	13.245	-2.438	0.056	0.142	0.802
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	938	0.120	16.672	-0.842	0.020	0.205	0.775
B S	gi_11386626_sp_O04379.1_AGO1_ARATH	939	0.122	14.345	-1.906	0.020	0.205	0.775
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	940	0.264	60.548	0.393	0.019	0.141	0.840
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	941	0.093	13.225	-0.240	0.019	0.141	0.840
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	942	0.087	9.620	-0.749	0.005	0.336	0.660
B H	gi_11386626_sp_O04379.1_AGO1_ARATH	943	0.149	27.012	-0.169	0.002	0.816	0.182
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	944	0.033	7.116	-0.132	0.001	0.900	0.099
B H	gi_11386626_sp_O04379.1_AGO1_ARATH	945	0.117	21.210	0.896	0.001	0.959	0.040
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	946	0.022	3.351	1.202	0.001	0.959	0.040
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	947	0.029	5.347	0.577	0.001	0.959	0.040
B W	gi_11386626_sp_O04379.1_AGO1_ARATH	948	0.121	29.197	0.823	0.001	0.900	0.099
B D	gi_11386626_sp_O04379.1_AGO1_ARATH	949	0.240	34.598	-0.660	0.004	0.514	0.481
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	950	0.311	54.262	-0.481	0.005	0.045	0.951
E N	gi_11386626_sp_O04379.1_AGO1_ARATH	951	0.494	72.395	-1.542	0.005	0.015	0.979
E N	gi_11386626_sp_O04379.1_AGO1_ARATH	952	0.597	87.372	-0.053	0.005	0.015	0.979
B F	gi_11386626_sp_O04379.1_AGO1_ARATH	953	0.087	17.441	0.173	0.005	0.015	0.979
E T	gi_11386626_sp_O04379.1_AGO1_ARATH	954	0.420	58.309	0.531	0.005	0.015	0.979
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	955	0.250	27.517	-1.125	0.858	0.002	0.139
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	956	0.497	71.675	-0.453	0.923	0.002	0.076
B G	gi_11386626_sp_O04379.1_AGO1_ARATH	957	0.205	16.173	1.024	0.923	0.002	0.076
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	958	0.036	6.500	-0.687	0.970	0.001	0.030
B Q	gi_11386626_sp_O04379.1_AGO1_ARATH	959	0.081	14.467	0.343	0.970	0.001	0.030
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	960	0.335	39.285	0.919	0.970	0.001	0.030
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	961	0.038	6.994	0.807	0.970	0.001	0.030
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	962	0.017	2.400	1.027	0.970	0.001	0.030
B N	gi_11386626_sp_O04379.1_AGO1_ARATH	963	0.087	12.795	-0.130	0.975	0.003	0.022
B N	gi_11386626_sp_O04379.1_AGO1_ARATH	964	0.197	28.782	-0.158	0.938	0.007	0.055
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	965	0.039	7.086	0.597	0.879	0.010	0.111
B C	gi_11386626_sp_O04379.1_AGO1_ARATH	966	0.018	2.513	0.760	0.717	0.014	0.269
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	967	0.060	12.907	0.322	0.660	0.049	0.291
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	968	0.059	8.239	-0.737	0.605	0.105	0.290
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	969	0.066	14.168	0.424	0.453	0.248	0.299
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	970	0.053	5.797	-0.196	0.321	0.252	0.427
B R	gi_11386626_sp_O04379.1_AGO1_ARATH	971	0.158	36.136	-0.605	0.216	0.235	0.548
B C	gi_11386626_sp_O04379.1_AGO1_ARATH	972	0.068	9.477	-0.961	0.118	0.150	0.732
B T	gi_11386626_sp_O04379.1_AGO1_ARATH	973	0.228	31.554	-1.082	0.052	0.084	0.864
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	974	0.341	78.181	-0.744	0.018	0.047	0.935
B S	gi_11386626_sp_O04379.1_AGO1_ARATH	975	0.120	14.041	-1.396	0.018	0.047	0.935
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	976	0.062	9.529	-0.726	0.020	0.205	0.775

B S	gi_11386626_sp_O04379.1_AGO1_ARATH	977	0.155	18.119	-1.465	0.021	0.279	0.699
B I	gi_11386626_sp_O04379.1_AGO1_ARATH	978	0.057	10.619	0.353	0.118	0.150	0.732
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	979	0.043	6.655	0.312	0.522	0.016	0.462
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	980	0.066	9.394	-0.044	0.858	0.002	0.139
B P	gi_11386626_sp_O04379.1_AGO1_ARATH	981	0.025	3.562	0.468	0.923	0.002	0.076
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	982	0.020	2.171	1.190	0.938	0.007	0.055
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	983	0.131	28.059	0.961	0.975	0.003	0.022
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	984	0.046	9.873	0.761	0.975	0.003	0.022
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	985	0.014	1.510	1.362	0.970	0.001	0.030
B H	gi_11386626_sp_O04379.1_AGO1_ARATH	986	0.155	28.249	0.399	0.970	0.001	0.030
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	987	0.159	29.095	1.479	0.988	0.000	0.012
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	988	0.021	2.336	1.294	0.988	0.000	0.012
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	989	0.016	1.763	1.034	0.988	0.000	0.012
E F	gi_11386626_sp_O04379.1_AGO1_ARATH	990	0.346	69.482	0.792	0.970	0.001	0.030
B R	gi_11386626_sp_O04379.1_AGO1_ARATH	991	0.165	37.716	0.760	0.970	0.001	0.030
B A	gi_11386626_sp_O04379.1_AGO1_ARATH	992	0.021	2.270	0.420	0.923	0.002	0.076
B R	gi_11386626_sp_O04379.1_AGO1_ARATH	993	0.194	44.540	0.272	0.858	0.002	0.139
E F	gi_11386626_sp_O04379.1_AGO1_ARATH	994	0.350	70.165	0.352	0.802	0.014	0.185
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	995	0.099	21.156	0.525	0.751	0.050	0.199
B M	gi_11386626_sp_O04379.1_AGO1_ARATH	996	0.058	11.566	0.752	0.502	0.102	0.396
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	997	0.417	72.867	1.149	0.428	0.171	0.402
E P	gi_11386626_sp_O04379.1_AGO1_ARATH	998	0.537	76.186	-0.452	0.278	0.093	0.628
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	999	0.425	74.213	-0.436	0.113	0.043	0.844
E T	gi_11386626_sp_O04379.1_AGO1_ARATH	1000	0.418	57.935	-1.434	0.053	0.043	0.903
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	1001	0.393	46.024	-1.071	0.058	0.017	0.925
E D	gi_11386626_sp_O04379.1_AGO1_ARATH	1002	0.657	94.630	-1.703	0.058	0.017	0.925
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	1003	0.540	63.347	-1.701	0.058	0.017	0.925
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	1004	0.428	33.707	-2.042	0.113	0.043	0.844
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	1005	0.513	60.088	-1.169	0.113	0.087	0.800
E M	gi_11386626_sp_O04379.1_AGO1_ARATH	1006	0.440	87.984	-1.483	0.118	0.150	0.732
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	1007	0.433	47.717	-1.650	0.125	0.227	0.648
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	1008	0.495	58.026	-1.602	0.125	0.227	0.648
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	1009	0.463	36.399	-1.501	0.125	0.227	0.648
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	1010	0.453	53.127	-1.686	0.118	0.150	0.732
E M	gi_11386626_sp_O04379.1_AGO1_ARATH	1011	0.331	66.313	-2.026	0.118	0.150	0.732
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	1012	0.486	53.557	-1.844	0.113	0.087	0.800
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	1013	0.531	121.576	-1.294	0.191	0.086	0.723
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	1014	0.541	42.616	-2.235	0.184	0.043	0.773
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	1015	0.382	30.063	-2.149	0.268	0.043	0.689
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	1016	0.480	37.784	-1.606	0.268	0.043	0.689
E M	gi_11386626_sp_O04379.1_AGO1_ARATH	1017	0.443	88.724	-1.462	0.268	0.043	0.689
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	1018	0.424	46.747	-1.263	0.455	0.046	0.498
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	1019	0.365	28.710	-1.838	0.455	0.046	0.498
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	1020	0.543	124.393	-1.434	0.561	0.047	0.393
E S	gi_11386626_sp_O04379.1_AGO1_ARATH	1021	0.471	55.166	-1.339	0.430	0.016	0.555
E T	gi_11386626_sp_O04379.1_AGO1_ARATH	1022	0.447	61.999	-1.701	0.430	0.016	0.555
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	1023	0.441	100.989	-1.130	0.430	0.016	0.555
E G	gi_11386626_sp_O04379.1_AGO1_ARATH	1024	0.429	33.739	-1.220	0.455	0.046	0.498
E P	gi_11386626_sp_O04379.1_AGO1_ARATH	1025	0.433	61.514	-1.363	0.354	0.048	0.598
E N	gi_11386626_sp_O04379.1_AGO1_ARATH	1026	0.473	69.306	-1.024	0.455	0.046	0.498
E V	gi_11386626_sp_O04379.1_AGO1_ARATH	1027	0.335	51.443	-1.221	0.455	0.046	0.498
E N	gi_11386626_sp_O04379.1_AGO1_ARATH	1028	0.524	76.640	-1.193	0.522	0.016	0.462
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	1029	0.380	41.920	-1.314	0.522	0.016	0.462
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	1030	0.398	43.849	-0.936	0.622	0.015	0.363
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	1031	0.241	36.996	-0.929	0.622	0.015	0.363
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	1032	0.524	119.973	-0.089	0.622	0.015	0.363
E P	gi_11386626_sp_O04379.1_AGO1_ARATH	1033	0.485	68.807	-0.297	0.522	0.016	0.462
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	1034	0.183	33.507	-0.712	0.339	0.016	0.645
E P	gi_11386626_sp_O04379.1_AGO1_ARATH	1035	0.399	56.604	-0.570	0.257	0.016	0.727
E A	gi_11386626_sp_O04379.1_AGO1_ARATH	1036	0.550	60.654	0.465	0.339	0.016	0.645
B L	gi_11386626_sp_O04379.1_AGO1_ARATH	1037	0.091	16.607	-0.105	0.339	0.016	0.645
B K	gi_11386626_sp_O04379.1_AGO1_ARATH	1038	0.201	41.346	0.070	0.339	0.016	0.645
E E	gi_11386626_sp_O04379.1_AGO1_ARATH	1039	0.698	121.958	0.734	0.430	0.016	0.555
E N	gi_11386626_sp_O04379.1_AGO1_ARATH	1040	0.399	58.428	0.930	0.339	0.016	0.645
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	1041	0.045	6.963	0.301	0.339	0.016	0.645
E K	gi_11386626_sp_O04379.1_AGO1_ARATH	1042	0.419	86.271	0.853	0.181	0.016	0.803
E R	gi_11386626_sp_O04379.1_AGO1_ARATH	1043	0.477	109.256	0.270	0.053	0.043	0.903
B V	gi_11386626_sp_O04379.1_AGO1_ARATH	1044	0.142	21.749	0.585	0.021	0.279	0.699
B M	gi_11386626_sp_O04379.1_AGO1_ARATH	1045	0.053	10.625	-0.663	0.023	0.655	0.322
B F	gi_11386626_sp_O04379.1_AGO1_ARATH	1046	0.116	23.361	0.172	0.021	0.756	0.223
B Y	gi_11386626_sp_O04379.1_AGO1_ARATH	1047	0.155	33.188	-0.314	0.023	0.655	0.322
B C	gi_11386626_sp_O04379.1_AGO1_ARATH	1048	0.215	30.214	-1.557	0.005	0.045	0.951

Figure S6. Multiple sequence alignment of Arabidopsis (At), Rice (Os) and Maize (Zm) AGO7. The yellow highlighted regions are the “DDH” catalytic amino acid residues. No consensus “S” or “T” residue could be found among these three proteins in the region to the C terminal side of the catalytic histidine residue.


ZmAGO7 MEYERG-----DRAKRGRGGGGGGGGGGGRGGGGEYGRQHGGAYGGGGWHGHDEGGGYGG
AtAGO7 MEEKTH-----HHHHSTNKHI-------------------PSSKSRTPLL----------
OsAGO7 MEGEREGVVAKNEDNAGGGGGGLGTGGNGGGGGG------GSANGRRRWR----------
ZmAGO7 GRGGGGYYHQ--GPRGRGGWRGPGAGGS-QAYGSGGGRAWAPGPGEGRGLGVGREYAPVR
AtAGO7 ---HKPYHHH--VQTNPPPFLLH--PSS-HQNLNLVAS---NLPSSYYYYYYCYFYSQFH
OsAGO7 GGGSSGYRQHPIIQAYPALLPLPINGATGHAHINGAVSLPLPLP----------PPVL
ZmAGO7 RAAPAPAPAPREVATAPMVK-----EAPSS----------SGSVGYDAYFSVSDESM
AtAGO7 NSLPPPPPPHLLPLSPPL--------PPLL----------P--LPPPHSMTRFHKSL
OsAGO7 LYLQPPPPPPLLPLLPKVAAATFYGKPPKAADAAPRGSMWKHRPSKKPPPHAITAALLPL
ZmAGO7 SSDVDEMSASSWYLNRERITSSELAGVEPLASTLAATSSVGTRVPMQRPDCGGALSQAKV
AtAGO7 PV-SQVVERKQQHQQKKKI---QVSNNKVSGSIAIEEAAL---VVAKRPDFGG-QDGSVI
OsAGO7 PRDGKALQEKIFFANERKTSEKEVNHVDTHEKFTVAPLDN---AIARRPDMGG-VEGAEI
ZmAGO7 KLLVNHFIVNYQKVSTIFHYDINIKLDEASSKASGKELSKAEFLSVKDELFRESSLRRLS
AtAGO7 YLLANHFLVKFDSSQRIYHYNVEI------SPQPSKEIARM----IKQKLVETDRNSFSG
OsAGO7 PLSANHFLVQFDPGQKIFHYNVDI------SPRPSKETARM----IKKKLVEENPSVLSG
ZmAGO7 SCVAYDGGRNLFTSAE----------LPAGLFRVRVRS----------KAYIVSVD
AtAGO7 VVPAFDGRQNIYSPVEFQGDRLEFFVNLPIPSCKAVMNYGDLREKQPQKKIEKLFRVNMK
OsAGO7 SQPAFDGRKNLYSPVRFQEDRVEFFVSLPVALARCSVVKEDTGHMLDKQKL-KTFKVNVR
ZmAGO7 LKKQLPLSQLSD--------LPVPREVLQGLDVVVREASRWRKVILGRGFYSPS--SSID
AtAGO7 LVSKFDGKEQRK---EGEDWAPLPPEYIHALDVILRENPMEKCTSIGRSFYSSSMGGSKE
OsAGO7 LVSKLCGEDLNKYLNEDKDGIPLPQDYLHALDVVLREGAMESSILVGRSLYARSMGEARD
ZmAGO7 IGQGVVAMKGTQQTLKYTQQGLNLCVDYSVMPFYKAGPVMDLVHKIVGYLDYRTTLNKRQ
AtAGO7 IGGGAVGLRGFFQSLRHTQQGLALNMDLSITAFHESIGVIAYLQK---RLEFLTDLPRNK
OsAGO7 IGGGAVGLRGFFQRLRPTKQGLALNVDLSLSAFHESTGIISYLQK---RCDFLKDLPQKK
ZmAGO7 MENLVDE--------LKGRRVTVIHRRTNQKYTVQGLTPLPASQMTFVDAESGQTKCLVE
AtAGO7 GRELSLEEKREVEKALKNIRVFVCHRETVQRYRVYGLTEEITENIWFPDRE-GKYLRLMS
OsAGO7 TRALAEEEHREVEKALKNIRVFVCHRETNQRYHVHSLTKETTENLKFRDRS-GKDLMVVD
ZmAGO7 YYAQKHGIVIEYQMLPCLDLSKSKDKPNHVPIELCTLLEGQRFPKANLDKNSGRILKGKA
AtAGO7 YFKDHYGYEIQFKNLPCLQISRA--RPCYLPMELCMICEGQKFLGKLSDDQAAKIMKMGC
OsAGO7 YFKEHYNHDIQFRNLPCLQIGRS--KPCYVPMELCVVCEGQKFLGKLSDEQTSKILKMGC
ZmAGO7 LIPASNRRKEILD-LVNASDGPCRGEIAQRFGISLDLRMTEVTGRILPPPNLKLGASNGQ
AtAGO7 QKP--NERKAIIDKVMTGSVGPSSGNQTREFNLEVSREMTLLKGRILQPPKLKL------
OsAGO7 ERP--SERKGIIKGVVKGAFHARSDTYADQFSLQVSKHMTKLSGRVLLPPKLKLGSSGRI
ZmAGO7 TSKFSIDQNCQWNLVKKRLVEGRDLQCWGIVDFSAEPSDPQQEPLNGRMFIEKIVRKCCE
AtAGO7 --------DRPRNLKESKVFKGTRIERWALMSIGGSSDQKSTIP----KFINELTQKCEH
OsAGO7 KDITPDRFDRQWSFLDSHVAEGSKIKSWALISFGGTPEQHFCIT----KFVNQLSNRCEQ

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ZmAGO7          LGIRMNSN----PCFVHKSKMAVLSDPHRLQEELNKAKQAAVSKKQRLQLLFCPMSEQHP
AtAGO7          LGVFLSKNTLSSTFF---EPSHILNNISLLESKLKEIQRAASNN---LQLIICVMEKKHK
OsAGO7          LGILLNKKTIISPIF---ERIQLLNNVGILEGKLKKIQEAASGN---LQLLICVMERRHQ
                **: :..:    . *   .   :*.:   *: :*:: : ** .:   ***::* *. .*

ZmAGO7          GYKTLKLICDTQLGIMTQCFLGDRANKPNGQDQYMTNLALKINGKLGGSNVQLFDSLPR-
AtAGO7          GYGDLKRISETRIGVVTQCCLYPNITKLSS--QFVSNLALKINAKIGGSMTELYNSIPSH
OsAGO7          GYADLKRIAETSIGVVTQCCLYSNLSKLTS--QFLTNLALKINAKLGGCNIALYSSFPCQ
                **  ** *.:* :*::*** *  . .* ..  *:::*******.*:**.   *:.*:*

ZmAGO7          -----VGGAPFMFIGADVNHPSPGNVESPSIAAVVASIN-SGVSKYVTRIRAQPHRCEVI
AtAGO7          IPRLLRPDEPVIFMGADVTHPHPFDDCSPSVAAVVGSINWPEANRYVSRMRSQTHRQEII
OsAGO7          IPRIFLSEEPVMFMGADVTHPHPLDDSSPSVVAVVASMNWPSANKYISRMRSQTHRKEII
                     *.:*:****.** * :  ***:.***.*:* . ...*::*:*:*.** *:*

ZmAGO7          QQLGEICLELIGVFEKRNRVKPQKIIYFRDGVSDGQFDMVLNEELADLEKAI-KVGGYAP
AtAGO7          QDLDLMVKELLDDFYKAVKKLPNRIIFFRDGVSETQFKKVLQEELQSIKTACSKFQDYNP
OsAGO7          EQLDVMAGELLEEFLKEVGKLPSRIIFFRDGVSETQFYKVLKEEMHAVRTTCSRYPGYKP
                ::*. :  **:  * *     *..**:******: **  **:**:  : .:  .  .* *

ZmAGO7          TVTVIVAKKRHHTRLFPKDPSQPQTK--NGNVPPGTVVDTGVVDPSAYDFYLCSHAGILG
AtAGO7          SITFAVVQKRHHTRLFRCDPD-------HENIPPGTVVDTVITHPKEFDFYLCSHLGVKG
OsAGO7          LITFIVVQKRHHTRLFHRERNGSSSHYSDQNIPPGTVVDTVITHPREFDFYLCSHWGTKG
                 :*. *.:********  : .         *:******** :. *  :******* *  *

ZmAGO7          TSRPTHYYSLVDEHGFRSDDLQKLVYNLCFVFARCTKPVSLATPVYYADLAAYRGRLYYE
AtAGO7          TSRPTHYHILWDENEFTSDELQRLVYNLCYTFVRCTKPISIVPPAYYAHLAAYRGRLYIE
OsAGO7          TSRPTHYHVLWDENNFRSDEVQQLIHNLCYTFARCTRPVSLVPPAYYAHLAAYRGRLYLE
                *******: * **: * **::*.*::***:.*.***.*:*:..*.*** ********* *

ZmAGO7          AAMMPSHQRGTGSASSGSSAGTFGVTNFPRLHKDVENNMFFI
AtAGO7          RS--SESNGGSMNPSSVSRVGPPKTIPLPKLSDNVKNLMFYC
OsAGO7          RS-----------DTTMYRVSPLQTVPLPKLRDNVKRLMFYC
                 :            ::   ...  .  :*.* .:*:. **:
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Figure S7. Multiple sequence alignment of Drosophila AGO1 and 2 (DmAGO1 and 2), Arabidopsis AGO1, 4 and 7(AtAGO1,4 and 7), and Human AGO2 (HsAGO2).

The yellow highlighted residues are the "DDH" catalytic amino acid residues. The green highlighted residues are the phosphorylated residues according to the Arabidopsis (Zulawski et al., 2013) and animal protein phosphorylation (Dinkel et al., 2011) databases.

No consensus "S" or "T" residue could be found among these proteins in the region to the C terminal side of the catalytic histidine residue.


PIWI domain
DmAGO2
AtAGO4
AtAGO7
AtAGO1
HsAGO2
DmAGO1

```
DmAGO1          EHDSGEGSHQS----GCSEDR------TPGAMARAITVHADTKKVMYFA
                          *                        .     . *::
```

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