Functional analysis of the *Arabidopsis* miR159 family

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Statement of Authorship

The research carried out in this thesis was conducted at the Australian National University and CSIRO Plant Industry between March 2006 and December 2009. I declare that this submission is my own work, and to the best of my knowledge contains no material previously published or written by another person nor material which has been accepted for the award of any degree or diploma of a university or other institution of higher learning except where due acknowledgement has been made.

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Presentations and Publications

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Abbreviations

APS	ammonium persulfate
bp	base pair
CTAB	Cetyl trimethylammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dsRNA	double stranded RNA
EDTA	ethylenediamine tetra-acetic disodium salt
FA	Formaldehyde
GUS	B-glucuronidase
IAA	isoamyl alcohol
IPTG	isopropyl-C-D-thiogalactoside
kb	kilobase-pairs
LB	Leuria-Bertani medium
LS LB	Low Salt Leuria-Bertani medium
min	minute(s)
miRNA	microRNA
MOPS	N-morpholino propane-sulfonic acid
mRNA	messenger RNA
nt	nucleotide
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RT-PCR	reverse transcriptase polymerase chain reaction
qRT-PCR	quantitative real time polymerase chain reaction
qSL-PCR	quantitative stem-loop real time polymerase chain reaction
s	second(s)
SDS	sodium dodecyl sulphate
SSC	Saline sodium citrate
sRNA	small RNA
siRNA	small interfering RNA
ssRNA	single stranded RNA
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	Tetramethylenediamine
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indoyl-C-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indoyl-C-D-glucuronic acid

Abstract

The deeply conserved *MIR159* gene family contains three members in *Arabidopsis*. Bioinformatics predict all *Arabidopsis* miR159 members to target seven genes of the *GAMYB-like* family. Several other genes unrelated to *GAMYB-like* also contain predicted miR159 target sites. However using a loss-of-function *mir159ab* mutant, it was found that only *MYB33* and *MYB65* are relevant *in vivo* miR159 targets. The *mir159ab* mutant displayed pleiotropic developmental defects, consistent with specific deregulation of *MYB33* and *MYB65*. GUS reporter gene constructs demonstrated that widespread transcriptional domains of *MIR159a* and *MIR159b* overlap with similarly widespread transcriptional domains of *MYB33* and *MYB65,* except in anthers, where *MYB33* and *MYB65*, but not *MIR159a* and *MIR159b* appear be expressed. By contrast, other targets of miR159 appear to be predominantly transcribed in anthers and pollen, suggesting the functional specificity of miR159a/miR159b is largely due to differences in transcriptional domains of miR159 target genes. Removing *MYB33* and *MYB65* from *mir159ab* suppressed all pleiotropic phenotypes of the *mir159ab* mutant, explicitly demonstrating the biologically relevant targets of miR159a and miR159b are *MYB33* and *MYB65*.

The possibility that miR159c may regulate anther/pollen transcribed miR159 target genes was investigated. It was found that despite an expression domain overlapping with these targets, both low expression and processing renders miR159c unable to regulate these targets. Investigation of *MYB101*, a target that is co-expressed with *MIR159c*, revealed it to be largely independent of miR159 regulation, indicating that miR159c regulation of other targets may have had importance previously, but now likely represents an obsolete regulatory module. The discrete functional specificity of the *Arabidopsis* miR159 family is narrower than predicted by overexpression studies or bioinformatics. These results reflect similar findings in metazoan systems, where removal of single targets can rescue the pleiotropic phenotypes of miRNA loss of function mutants. Thus a greater functional specificity of miRNAs than predicted by bioinformatics or suggested by overexpression studies is an emergent and unifying theme in both plant an animal studies.

Chapter 1

Introduction

It's a world of laughter, a world of tears
it's a world of hopes, and a world of fears
there's so much that we share
that's its time we're aware
it's a small world after all

-Robert and Richard Sherman.
(By commission to Walt Disney)

1.1 Discovery of regulatory small RNA

The discovery of regulatory small RNA (sRNA) has led to a paradigm shift in our understanding of gene regulation. Although the notion that RNA may modulate protein synthesis was introduced nearly 50 years ago (Jacob and Monod, 1961), a protein-centric depiction of gene regulation has since prevailed. However this view cannot adequately describe the complexity required of higher organisms to control gene expression (Sharp, 2009). Only recently we have began to appreciate how intrinsic regulatory sRNA is to the control of virtually every genetic process.

The existence of endogenous small ribo-oligonucleotides was first demonstrated in 1987 (Plesner et al., 1987). Although the notion that such small RNAs could provide regulatory functions did not escape the author's attention, it was not until 1993 that the first small regulatory RNA, the *lin-4* miRNA, was identified (Lee et al., 1993). Subsequent work by Waterhouse et al (1998) and Fire et al (1998) discovered that double stranded RNA induced post transcriptional gene-silencing, also termed RNA interference (RNAi), proposed to act through complimentary small RNAs (Waterhouse et al., 1998). Identification of 21-25 nt sRNAs (Hamilton and Baulcombe, 1999) as the effectors of RNAi led to an explosion in the discovery of sRNAs in plants and animals, revealing a vast array of these molecules that appeared to function as negative regulators of gene expression.

This thesis concerns microRNAs (miRNAs), the second most abundant sRNA class in *Arabidopsis*. A taxonomic description of all sRNA classes and functions is beyond the scope of this introduction, but extensive reviews can be found elsewhere (Chapman and Carrington, 2007; Chen, 2009; Voinnet, 2009). However distinctions between sRNA classes are becoming progressively blurred, and in some cases overlap in terms of sRNA origin, biogenesis and function. Therefore this introduction will focus on plant miRNAs and later miR159, but will introduce concepts from other sRNA classes where they may be relevant to plant miRNAs and the scope of this thesis.

1.2 miRNA biogenesis.

MIRNA genes are transcribed by RNA polymerase II, usually in regions between protein coding genes (Xie et al., 2005) (Fig 1.1). Methyl capped and polyA tailed primary transcripts (called the pri-miRNA) form secondary structures, and recently DAWDLE (DDL) proteins have been identified in *Arabidopsis* that appear to help stabilise pri-miRNA structures (Yu et al., 2008). Pri-miRNAs characteristically contain a dsRNA stem-loop that can range from 50-250 nt in length (Reinhart et al., 2002) but ~70 nt is most frequent (Bologna et al., 2009). These stem-loops have imperfect complementarity (Meyers et al., 2008), containing several bulges, and the mature miRNA sequence resides within the stem.

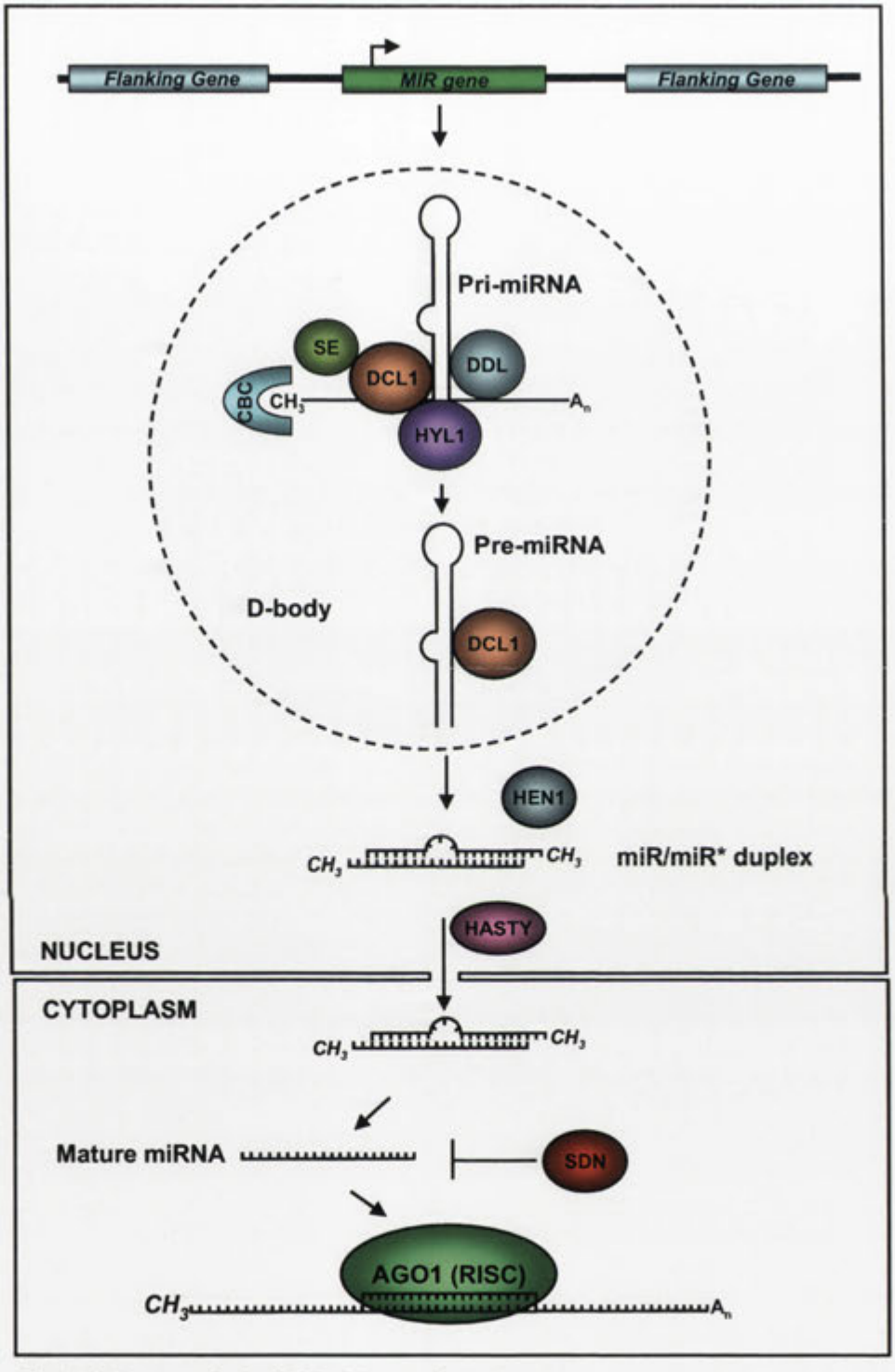


Fig 1.1 The canonical miRNA biogenesis pathway.
The location and relationship of known miRNA pathway components is shown. Abbreviations: CBC, nuclear cap-binding complex; SE, SERRATE; DCL1, DICER-LIKE1; DDL, DAWDLE; HYL1, HYPONASTIC LEAVES1; HEN1, HUA-ENHANCER1; SDN, SMALL RNA DEGRADING NUCLEASE; AGO1, ARGONAUTE1; RISC, RNA INDUCED SILENICING COMPLEX. See text for details. Exceptions to this generalised pathway are noted in the text.

Excision of the miRNA begins with cleavage of the stem-loop at its base by a DICER-LIKE (DCL) protein (Kurihara and Watanabe, 2004; Park et al., 2002). There are four DCL proteins in *Arabidopsis*, but DCL1 is predominantly responsible for processing miRNAs (discussed further below). This excised stem-loop is called the pre-miRNA, or interchangeably, the miRNA stem-loop. DCL1 physically interacts with HYPONASTIC LEAVES1 HYL1 (Han et al., 2004; Vazquez et al., 2004) and SERRATE (Lobbes et al., 2006; Yang et al., 2006) proteins that assist miRNA processing. Cap-binding complex (CBC) proteins also assist in processing some, but not all miRNAs (Gregory et al., 2008; Laubinger et al., 2008). For example miR172 levels appeared unchanged in CBC mutant lines in contrast to most other miRNAs assayed (Laubinger et al., 2008). Both CBC and SE proteins additionally function in mRNA splicing (Laubinger et al., 2008).

Recent evidence has shown DCL1 does not process all pri-miRNAs (Fahlgren et al., 2007; Rajagopalan et al., 2006; Vazquez et al., 2008). Of the four DCL paralogues, it has been shown that DCL4 can also process miRNAs. This has been partially explained by the affinities of different DCLs for distinct dsRNA templates. In this regard, DCL4 has been demonstrated to process long dsRNA of perfect complimentarity required for *trans* acting small RNAs (tasiRNA) biogenesis (see below) or silencing of exogenous dsRNA (Deleris et al., 2006; Fusaro et al., 2006; Yoshikawa et al., 2005). It has also been demonstrated that DCL4 processes miRNAs that are evolutionary young, that is, miRNA genes that have long hairpin structures with high dsRNA complimentarity, proposed hallmarks of recent evolution through inverted duplication events (Allen et al., 2004; Fahlgren et al., 2007; Rajagopalan et al., 2006; Vazquez et al., 2008). This model predicts miRNAs can evolve from inverted duplication of protein coding genes that give

rise to long fold-back dsRNA structures. If siRNAs produced from these render a selective advantage, the loci that produce them can become ingrained in regulatory networks, eventually forming miRNA genes and acquiring DCL1 processing by accumulation of bulges. This has been supported by deep sequencing data revealing differing patterns of sRNA accumulation for anciently conserved and recent evolved miRNA loci (Fahlgren et al., 2007; Rajagopalan et al., 2006). Nevertheless the comparatively high importance of DCL1 in miRNA biogenesis and development is exemplified by embryonic lethal phenotypes seen in *dcl1* mutants (Jacobsen et al., 1999), in contrast to far less drastic developmental phenotypes seen even in *dcl2/dcl3/dcl4* triple mutants (Henderson et al., 2006).

It appears that pri-miRNA processing, or at least storage of processing machinery occurs in specialised regions of the nucleus called dicing-bodies (D-bodies) (Fang and Spector, 2007; Song et al., 2007) demonstrated by co-localisation of fluorescently tagged DCL1, HYL1 and SE proteins in discrete nuclear regions. D-bodies appear distinct from Cajal bodies- other discrete nuclear regions containing DCL3 and further sRNA pathway components associated with DNA methylation and chromatin remodelling (Li et al., 2006; Pontes et al., 2006). Following excision of the pre-miRNA, DCL1/DCL4 makes further cuts to liberate a 20-24 nt dsRNA duplex with 2 nt 3' overhangs (Kurihara and Watanabe, 2004). This duplex contains the miRNA sequence, and the near reverse complement is called the miRNA*. It has been recently shown for the miR159/319 families that have long stem-loops, cleavage by DCL1 begins at the end of the stem-loop furthest from the pre-miRNA base (Bologna et al., 2009), whereas shorter pre-miRNAs appear to be cut successively from the base towards the loop, as

described for miR163 (Kurihara and Watanabe, 2004), miR164/miR166 (Kurihara et al., 2006), miR168 (Vaucheret et al., 2006) and miR172 (Bologna et al., 2009).

A consensus biochemical step shared by all plant sRNAs is their 3-O-methylation. This is carried out in the nucleus by HUA ENHANCER-1 (HEN1) on miRNA/miRNA* duplexes (Yu et al., 2005). This step appears important but not critical, as *hen1* mutants are viable, despite no evidence of redundant partners, and the fact that miRNAs can still accumulate (although with reduced abundance) in *hen-1* mutants (Park et al., 2002). HEN1 mediated methylation appears to protect sRNAs from uridylation and subsequent degradation. Transport of miRNAs to the cytoplasm is mediated by the HASTY protein (Park et al., 2005), but it is uncertain whether the miRNA/miRNA* or single stranded molecules (or both) are transported. However accumulation of not all miRNAs appears to be affected in *hasty* mutants, indeed some evidence suggests that not all miRNA action is cytoplasmic (see below), and therefore may not require HASTY mediated nuclear export.

After or perhaps during entry to the cytoplasm, the miRNA strand of the miRNA/miRNA* duplex is loaded into the ARGONAUTE (AGO) protein (described below). At this stage the mature miRNA is subject to regulation by SMALL RNA DEGRADING NUCLEASES (SDNs) (Ramachandran and Chen, 2008). SDNs act to specifically degrade mature miRNAs, but it is unclear whether this occurs during or after loading into AGO proteins. This function appears widely important as *sdn* mutants show pleiotropic phenotypes and over-accumulate miRNAs.

AGO proteins are common to plant and animal sRNA pathways, and carry out the negative regulatory function of miRNAs and siRNAs (reviewed in (Vaucheret, 2008)). In *Arabidopsis* there are ten AGO proteins, but AGO1 appears to be the predominant form required for miRNA function; *ago1* null alleles show extreme developmental defects, in contrast to milder defects found in knockouts of other *AGO* genes (Bohmert et al., 1998; Vaucheret, 2008). The proliferation of AGOs in *Arabidopsis* is reflected by their functional diversity, where different members appear to be associated with different sRNA pathways. This is further reflected in the distribution of different sRNAs in various AGO proteins, where for example, more than 80% of AGO1 containing miRNA sequences begin with a 5' Uracil, whereas AGO4, associated with heterochromatic gene silencing, tends to favour 5' Adenosines associated with siRNAs (Mi et al., 2008; Montgomery et al., 2008). This selectivity has been suggested to be an intrinsic feature of AGO proteins, where changes to 5' nucleotides of artificial miRNAs can change their distribution within AGOs and their function (Mi et al., 2008). However other evidence indicates that the 5' nucleotide does not influence AGO loading; in a recent experiment the main determinant of AGO loading appeared to be biases in the 5'thermodynamic stability of miR/miR* duplexes (Eamens et al., 2009). Functional roles for all argonautes have not been established, and only AGO1, AGO4, AGO6, AGO7 and AGO10 have been functionally characterised (reviewed in Vaucheret 2008).

AGO proteins that contain a sRNA are referred to as RISC- the RNA Induced Silencing Complex (Hammond et al., 2000). It has been frequently stated that RISC complexes "scan" mRNAs until target sites are found, perhaps similar to mechanisms used to locate translation initiation sites (Kozak, 1999). However in plants (or animals) there

has been no evidence presented to support such a mechanism. For animal RISC complexes, experiments that blocked scanning ability did not affect target down-regulation, and suggested RISC acts through a diffusion mechanism instead (Brown et al., 2005). Given there are structural and functional differences between animal and plant RISCs, it is currently unclear what mechanism plants RISCs may use to locate potential targets.

1.3 miRNA action

1.3.1 Cleavage of mRNA targets

The first form of target regulation demonstrated by plant RISCs was cleavage, shown to occur by "slicing" of the phosphodiester bond between nucleotides 10-11 of the miRNA binding site in the mRNA target. This has been demonstrated by northern analysis and recovery of miRNA guided cleavage fragments of mRNA targets by 5'-RACE. (Dunoyer et al., 2004; Llave et al., 2002; Palatnik et al., 2003) (reviewed in Jones-Rhoades et al 2006). The sequence requirements for cleavage have been experimentally tested using different approaches, and it has been found that a relatively high degree of complementarity is required between the miRNA and target. Specifically, positions 2-12 of the miRNA have been shown to be critical for target down-regulation (Schwab et al., 2005). In this region is has been demonstrated that only one mismatch between a target and a miRNA is tolerated for cleavage to occur, except at positions 10 and 11, where any mismatch abolishes cleavage. This region's importance has also been demonstrated by analysis of miRNA target site mutations, where for example it has been shown that silent single base mutations in *PHABULOSA* (*PHB*) genes can reduce their cleavage by miR165/166 (Mallory et al., 2004b). These experiments suggested that

like animal RISC complexes, strong pairing at the 3' end of the target seems particularly critical for their cleavage.

However unlike animal RISC complexes, it appears that high complementarity throughout the entire 20-24 nt region is also important. This has been elegantly demonstrated in experiments that have examined the closely related miR159/miR319 families, and found that miR159 is unable to regulate miR319 targets by cleavage due to lack of sufficient complementarity with *TCP* targets (Palatnik et al., 2007). In addition, single base mutations generated mismatches in the 5' end of *TCP*s targets rendered them resistant to miR319 down-regulation. These outcomes further demonstrated that high fidelity between plant miRNA and mRNA targets is required for their cleavage.

1.3.2 Translational repression

A second mode of target down-regulation by plant miRNAs occurs through translational repression of mRNA targets. Although initially this was demonstrated for only few targets, early clues of more general translational repression were provided by discrepancies observed between protein and message levels for several targets of miRNAs (Aukerman and Sakai, 2003; Bari et al., 2006; Chen, 2004; Gandikota et al., 2007). Further recent evidence has led to the claim that this mechanism might be widespread in plants; a mutant screen found the mRNA de-capping factor VARICOSE, and the microtubule severing enzyme KATANIN, both general cellular components implicated with translation, were required to repress the protein levels of both natural and artificially introduced miRNA targets (Brodersen et al., 2008).

Up until recently, it was commonly inferred for both animals and plants that translational repression can occur through low complimentary miRNA:target interactions, whilst cleavage occurs when there is high complimentarity between miRNAs/siRNAs and their targets. This notion derives from earlier findings in animal systems; perfect complimentarity siRNAs have been shown to cleave target mRNAs (Elbashir et al., 2001; Hammond et al., 2000; Yang et al., 2000), whereas miRNAs that predominantly reduce protein levels of their targets usually have several target mismatches (Doench and Sharp, 2004). In *Arabidopsis*, a recent finding that a low complimentary target of miR398 is only affected at the protein, but not message level (Dugas and Bartel, 2008), suggests that similar modes of operation may also occur in plants.

However other experiments that have assessed the possibility of protein down-regulation for low complementarity targets of plant miRNAs have found no evidence for this (Palatnik et al., 2007). In addition, close phenotypical similarities between miRNA over-expressors and knockout mutants of their target mRNAs supports the notion that similarly high sequence requirements are required for both cleavage and translational repression in plants (Alvarez et al., 2006; Aukerman and Sakai, 2003; Laufs et al., 2004; Mallory et al., 2004a; Schwab et al., 2006; Schwab et al., 2005). Therefore, although translational repression of a low complimentarity target has been demonstrated in *Arabidopsis*, there remains no clear evidence that such relatively low fidelity repression represents a widespread mode of action for plant miRNAs. In any case, it appears that target cleavage by miRNAs is developmentally more critical than translational repression. This is because mutations in *ago1* that abolish only

translational repression but maintain slicing are far less deleterious than mutations in *ago1* that abolish slicing (Brodersen et al., 2008).

Although translational repression must necessarily occur in the cytoplasm, there is some evidence that cleavage by miRNAs may also occur in the nucleus. This has been demonstrated by recovery of miRNA guided cleavage products for pri-miR172, and some other pri-miRNAs show evidence of cleavage in the centre of their miRNA sequences (German et al., 2008). Additionally AGO1, when transiently expressed in tobacco leaf cells as a fusion protein, was found to localise in both the cytoplasm and nucleus (Fang and Spector, 2007). However the full extent to which cleavage by miRNAs may occur in the nucleus is still unknown. Alternately, it is possible that some pri-miRNA transcripts may escape into the cytoplasm where they can be cleaved by RISCs.

1.4 Different functions of plant miRNAs

1.4.1 Direct regulation of mRNA targets

Thus far the function of plant miRNAs has been implicated in the down-regulation of target mRNAs. Within this broad role, there are several different modes by which plant miRNAs have been proposed to operate. Firstly, it has been suggested that miRNAs can act like "switches" to completely abolish expression of targets transcribed in their same domain (Bartel, 2004). This is exemplified in the regulation of *PHB* transcription factors by members of miR165/166 that specify leaf polarity (Kidner and Martienssen, 2004). In such cases, although transcription of a target may be relatively constant between cell types, target expression can either be permitted or eliminated by the absence or presence respectively of the miRNA. Secondly, miRNAs have been

described to act as "tuners" where miRNAs are expressed in the same domain of the targets, but only dampen, rather than eliminate their expression (Flynt and Lai, 2008). Examples of this type of interaction have been shown for miR164 regulation of *CUC2* in *Arabidopsis* in controlling the leaf margin shape (Nikovics et al., 2006), and regulation of Petunia *BLIND* and *FISTULATA* floral identity genes by miRNAs related to *Arabidopsis* miR169 (Cartolano et al., 2007). In this mode of operation, miRNAs act as an additional regulatory layer to transcriptional control to directly modulate the expression level of targets. A third mode of operation has been illustrated where miRNAs are expressed in mutually exclusive domains to their targets (Kawashima et al., 2009), and serve as a regulatory "backup" to ensure expression of targets remains delineated if the target is transcribed outside its native domain.

1.4.2 Indirect regulation of targets

Another function of miRNAs that demonstrates cross-talk between different sRNA pathways is *trans*-acting small RNA (tasiRNA) production. Certain miRNAs have been shown to cleave target mRNAs that are subsequently copied by RNA-dependent RNA polymerase-6 (RDR6) into dsRNA substrates for processing by DCL4 (Allen et al., 2005). Therefore such miRNAs set the phase register for production of 21 nt small RNAs (Howell et al., 2007). In turn some of these siRNAs silence different genes from which they originate, hence their description as *trans*-acting siRNAs. Of interest, predominantly only miRNA generated *TAS* loci seem to be channelled into TAS pathways, whereas other cleaved miRNA targets appear not to form tasiRNAs, despite evidence that cleaved miRNA targets can be copied by RDR6 (Luo et al., 2009).

A major function of other sRNA classes has been shown to be methylation of DNA, but so far only one miRNA has been associated with methylation of target loci. This was demonstrated for miR165, where a mutation in the *PHB* target that rendered it cleavage resistant also correlated with reduced methylation within it's coding regions- but only on the mutant allele (Bao et al., 2004). Notably the region methylated was complementary to the *PHB* 3' cleaved mRNA fragement, and downstream of the miR165 target site, implying the miRNA itself is not methylating the target. This has prompted the suggestion that the 3' cleavage fragment itself may somehow guide methylation in this region (Voinnet, 2009). There is no other evidence that miRNAs may be involved in methylation, and the biological significance of *PHB* methylation by miR165 remains unclear.

1.5 miRNAs controlling development:

An early observation from the pleiotropic phenotypes of miRNA biogeneis mutants such as *dcl1*, *ago1*, *hyl1*, *hen1* and *hasty* was their obvious implication in development (Bohmert et al., 1998; Chen et al., 2002; Jacobsen et al., 1999; Lu and Fedoroff, 2000; Park et al., 2002; Telfer and Poethig, 1998). This is best exemplified where null mutants of *dcl1*, the key processing enzyme for miRNAs, were embryonic lethal, implicating the crucial role this enzyme (and the miRNA pathway) plays in early development. With the identification of miRNAs and potential targets, an explanation was provided for the developmental importance of miRNA pathway components; many of the earliest cloned miRNAs appeared to regulate transcription factors that were themselves controllers of developmental processes (Reinhart et al., 2002; Rhoades et al., 2002). The fact that many of the miRNAs controlling transcription factors were later found to be highly abundant and deeply conserved (Axtell and Bartel, 2005; Floyd and Bowman, 2004),

helped to reinforce the notion that miRNAs are key developmental regulators. More recent deep sequencing efforts across different species have revealed a greater number of miRNAs that control a diverse range of processes not necessarily developmentally related (Ding et al., 2009; Jian et al., 2009; Zhang et al., 2008). Therefore although some miRNAs may have deeply conserved functions in regulating basic developmental process, there is also a growing list of less conserved, usually less abundant miRNAs that have diverse roles.

Although it appears miRNAs can serve many regulatory roles, their absolute importance in plant development remains unclear. This is because although their *in vivo* importance has been insinuated by several means (briefly described below and also discussed later in this thesis), there have been remarkably few examples demonstrating that removal of miRNAs leads to developmental consequences (Cartolano et al., 2007; Nikovics et al., 2006; Sieber et al., 2007). Notably this applies not only for plant miRNAs, but metazoan miRNAs as well, where for example systematic deletion of 80 miRNA genes in *C.elegans* identified only four mutants with phenotypes different from wild-type (Miska et al., 2007).

1.6 Common approaches taken in determining miRNA importance

Overexpression of endogenous miRNAs has been extensively employed to demonstrate their involvement in developmental processes (Achard et al., 2004; Aukerman and Sakai, 2003; Chen, 2004; Laufs et al., 2004; Mallory et al., 2004a; Schwab et al., 2005; Wang et al., 2004). These studies have indicated a great diversity of roles for miRNAs, including, but not limited to, control of leaf development, floral identity, root development, and flowering time (reviewed in Mallory and Vaucheret., 2006). In

several cases, these experiments have been combined with analyses of their putative target's expression, to show such targets are down-regulated. These studies have often been extended to the recovery of miRNA guided cleavage products, in such cases unambiguously demonstrating that certain targets are cleaved by miRNAs. A further extension of this strategy has been to compare phenotypes of miRNA over-expressing transgenic plants with those of their target knockouts (Alvarez et al., 2006; Laufs et al., 2004; Mallory et al., 2004a; Schwab et al., 2006). These comparisons have revealed similar phenotypes, indicating overexpression of a miRNA can produce equivalent developmental consequences to removal of its target(s).

An additional approach taken is to study the effects of mutations in the miRNA target sites of mRNAs (Laufs et al., 2004; Mallory et al., 2005; Mallory et al., 2004b; Millar and Gubler, 2005; Nikovics et al., 2006; Ori et al., 2007; Palatnik et al., 2003; Palatnik et al., 2007; Wang et al., 2005). This has been performed by introducing miRNA resistant targets using transgenes, or otherwise by isolation of mutations within the miRNA target sites of endogenous genes. In fact several developmental mutants that predate plant miRNA discovery (Mathan and Jenkins, 1960; McConnell and Barton, 1998; Poethig, 1988) have subsequently been found to be miRNA target site mutants (Ori et al., 2007; Rhoades et al., 2002; Wu and Poethig, 2006). These approaches quite often reveal strikingly different phenotypical outcomes than overexpression of the equivalent miRNA (Williams et al., 2005; Xiao et al., 2007). For example overexpression of miR159 leads to anther defects, attributed to down-regulation of two targets, *MYB33* and *MYB65* (Achard et al., 2004), yet silent mutations in the miR159 target site of *MYB33* lead to pleiotropic developmental phenotypes (Millar and Gubler, 2005; Palatnik et al., 2003), suggesting deregulation of targets is more drastic than their

removal. Such contrasting results suggest that reliance on inferences from overexpression may understate or even misrepresent the developmental relevance of miRNAs.

1.7 *Arabidopsis* miR159

miR159 and regulation of its potential targets has been explored previously. Partially this is due to a historical coincidence, where before plant miRNAs had been identified, activation tagging studies identified a mutant (*jaw-1D*) (Weigel et al., 2000) that was subsequently found to over-expresses miR319 (Palatnik et al., 2003). With the discovery of miRNAs, miR319 was described as a close relative of miR159 (Jones-Rhoades and Bartel, 2004; Palatnik et al., 2003). Consequently, analysis of miR159 regulation was investigated concurrently with one of the first plant miRNAs (miR319) subject to detailed analysis. These studies indicated that miR319 and miR159 appeared to regulate different gene families; miR319 appeared to regulate *TEOSINTE BRANCHED CYCLOIPEDA PCF* (*TCP*) genes, while miR159 appeared to regulate *GAMYB-like* genes (Palatnik et al., 2003; Palatnik et al., 2007). More recently, analysis has revealed that miR319 and miR159 probably did have a common ancestor; they share considerable homology in their stem-loops dating back to the moss *Physcomitrella patens*, and an ancient *MYB* gene similar to the *Arabidopsis* miR159 *GAMYB-like* targets (see below) was shown to be cleaved by *P. patens* miR319 (Axtell et al., 2007).

1.8 miR159 targets

GAMYB transcription factors were originally identified in the Barley aleurone, where it was found the *HvGAMYB* gene encoded a gibberellin (GA) upregulated *MYB* gene,

which in turn activated the *α-Amylase* gene involved in starch degradation (Gubler et al., 1995). Subsequent investigations revealed Barley and Rice *GAMYB* (Kaneko et al., 2004; Murray et al., 2003), as well as *Arabidopsis GAMYB-like* genes (Millar and Gubler, 2005) all to be involved in anther development. Notably, all these genes contain similar miR159 sites, although *GAMYB* genes have expanded in *Arabidopsis* as there are seven members in the *GAMYB-like* family (*MYB33*, *MYB65*, *MYB81*, *MYB97*, *MYB101*, *MYB104*, *MYB120*) (Stracke et al., 2001). Other differences between *GAMYBs* (and possibly in their regulation) are suggested by their overexpression phenotypes; overexpression of *HvGAMYB* produces anther defects (Murray et al., 2003) but overexpression of *MYB33* produces no phenotype additional to wild-type (Millar and Gubler, 2005).

The importance of *MYB33* as a target of miR159 has been described using some of the widely employed approaches already mentioned above. Interestingly, overexpression of miR159a produced dramatically different outcomes in two experiments: Achard et al., (2004) were able to show down-regulation of *MYB33* transcript, anther defects and delayed flowering time. However, Schwab et al., (2005) were only able to find anther defects and could not detect changes to *MYB33* (or *MYB65*) levels. Differences in the overexpression efficiency between constructs and different genetic backgrounds used were suggested as reasons for these discrepancies (Schwab et al., 2005). These findings highlight potential complications associated with transgenic experiments.

As previously mentioned, mutation of the miR159 site in *MYB33* (using the *mMYB33* transgene) leads to widespread pleiotropic defects. This was also reflected using the miR159 resistant *MYB33* gene under its native promoter translationally fused to GUS; in *mMYB33:GUS* transgenic plants, expression of *mMYB33*:*GUS* was widespread, demonstrating the transcriptional domain of *MYB33* is also widespread (Fig 1.2). In contrast, *MYB33*:*GUS* was only detected in anthers, suggesting that miR159 regulation of *MYB33* is widespread, but is at insufficient levels or absent in anthers to allow MYB33 repression in this tissue (Millar and Gubler, 2005).

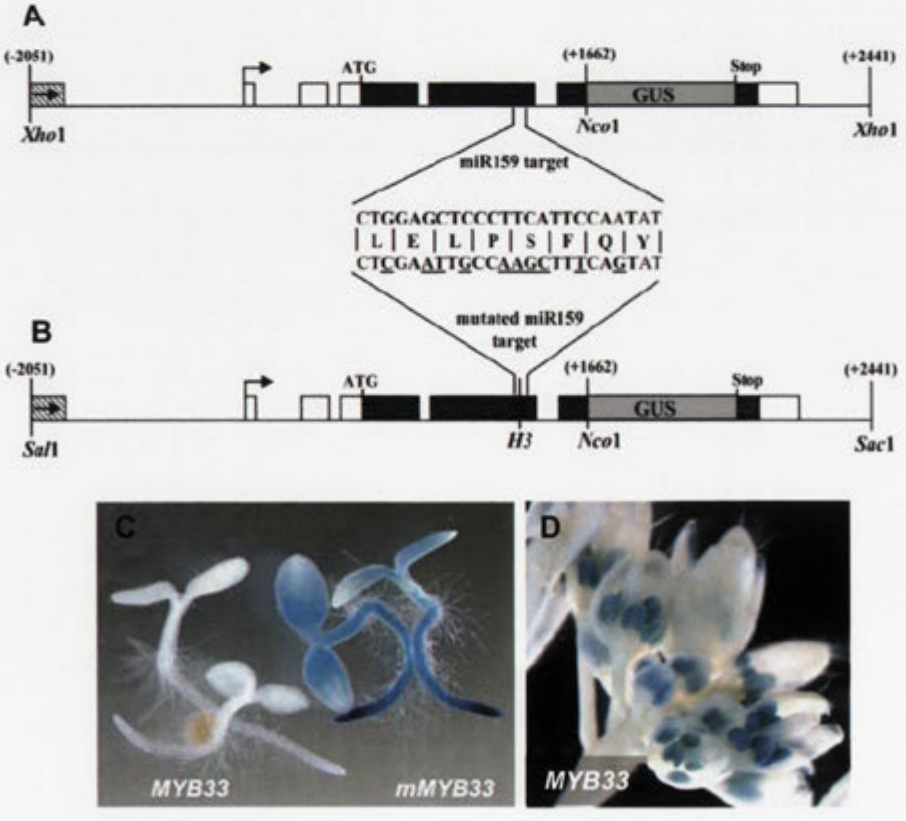


Fig 1.2 *MYB33* and *mMYB33* GUS in wild-type.

The genomic translational GUS fusions constructs used to analyse *MYB33:GUS* (A) and *mMYB33:GUS* (B) expression. (C) comparison of *MYB33:GUS* and *mMYB33:GUS* in 3 day old seedlings. MYB33:GUS protein was only detectable in anthers (D). Millar and Gubler (2005).

1.9 miR159 as a model for understanding plant miRNA function.

miR159 is a deeply conserved miRNA pre-dating the divergence of monocotyledonous and dicotyledonous plants. The miR159 target site in *GAMYB* has been conserved across these species. Furthermore, recent deep sequencing has revealed that miR159 is the most abundant miRNA in *Arabidopsis* (Backman et al., 2008). Clearly miR159 regulation of *MYB* (or other targets) appears intrinsic to plant gene regulation (Axtell et al., 2007), yet many questions remain unanswered; both in terms of miR159 regulation of it's potential targets, and also more broadly, in terms of general plant miRNA function.

Specifically, what genes are regulated by miR159? Although overexpression and other analyses have attempted to address this question, there is still no definitive answer to this question as it applies to miR159; or more broadly for other plant miRNAs. For example overexpression of miR159 led to down-regulation of the *OPT1* gene (among others), verified by recovery of miR159 directed *OPT1* cleavage products. However no cleavage products of *OPT1* were found in wild-type, demonstrating that *in vivo* concentrations and/or location of miRNAs and their targets can influence a miRNAs regulatory potential. Furthermore, this question is particularly relevant given that translational repression may be a major component of target down-regulation, yet previous studies have tended to focus on transcript analysis. Following on from this, is miR159 regulation of mRNA targets developmentally important? Again both

overexpression and analysis of miRNA resistant targets have suggested that basic developmental process rely on miRNAs to control them. Yet can we be sure these experiments, that employ artificial transgenes faithfully represent the *in vivo* function of miRNAs? As mentioned earlier, there have been remarkably few examples of miRNA loss-of-function approaches taken to addressing these questions. One of the proposed bottlenecks has been that redundancy within *MIRNA* gene families may conceal readily observable phenotypes that may only be apparent when several members are removed. If this is true, miR159 represents an ideal candidate to reveal miRNA function, as only three members exist in *Arabidopsis*, and their mature sequences differer by only 1-2 nt (Fig 1.3). Therefore this thesis will use a loss-of-function approach, combined with molecular and phenotypical analysis, to thoroughly investigate the miR159 family. This approach to plant miRNA functional analysis aims to determine the role miR159 plays in *Arabidopsis* gene regulation.

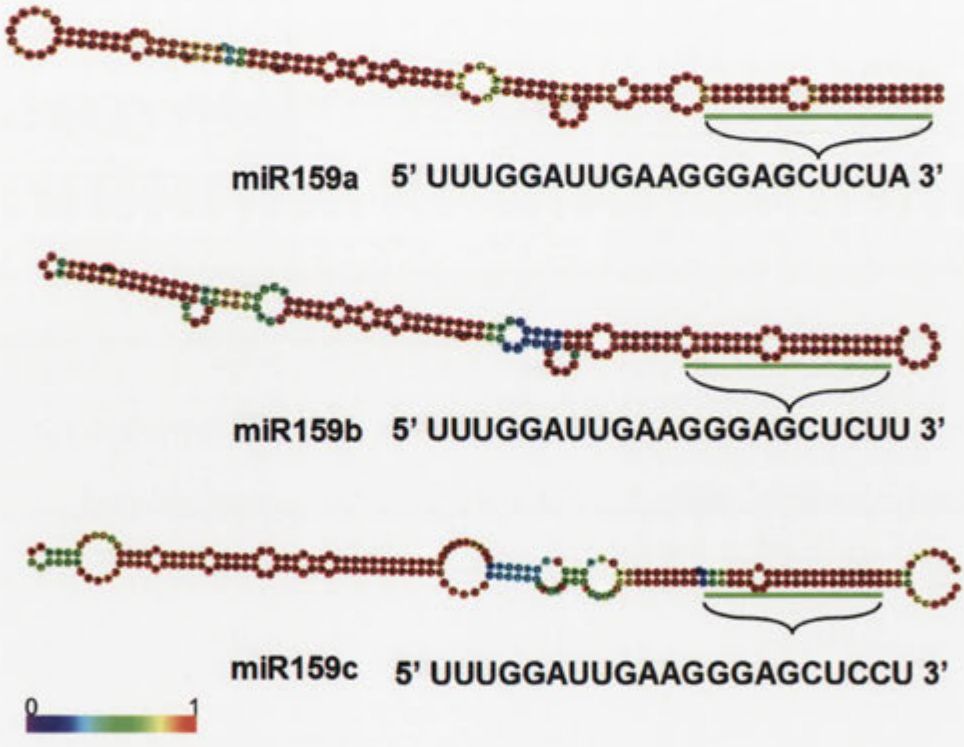


Fig 1.3 Predicted stem-loop structures of pre-miR159a, pre-miR159a and pre-miR159c and their mature miRNA squences.
Sequences were obtained from mirbase (Griffiths-Jones et al., 2008) and folded using mFold (Gruber et al., 2008). All mature miRNA sequences are processed from strong stem-loop pre-miRNA structures. Green bars show the region corresponding to the mature miRNA sequence. Base pairing probabilities are indicated by the heat map (blue=weak, red = strong).

Chapter 2

Materials and Methods

You could write up your materials and methods now, and as you go along
-Tony Millar, 2007

It would be a good idea to start writing your materials and methods now
-Tony Millar, 2008

You need to write your materials and methods
-Tony Millar, 2009

2.1 Bioinformatics

Identification of *Arabidopsis* T-DNA mutants was carried out using T-DNA express http://signal.salk.edu/cgi-bin/tdnaexpress (Alonso et al., 2003). Predicted stem-loop sequences of *MIR159* genes were obtained from miRbase http://www.mirbase.org/ (Griffiths-Jones et al., 2008). Sequence analysis of *MIR159* genes was carried out using NCBI pairwise blast http://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul et al., 1990) and clustalW2 http://www.ebi.ac.uk/Tools/clustalw2/index.html (Larkin et al., 2007). Sequencing analysis, vector design, and scale diagrams of *MIR159* genomic regions were performed using VectorNTi software (Invitrogen, Carlsbad, CA). Analysis of RNA folding was performed using mFold http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi (Gruber et al., 2008). Gene expression clustering analysis was performed using genevestigator https://www.genevestigator.com/gv/index.jsp (Hruz, 2008).

2.2 Growth of *Arabidopsis*

Arabidopsis thaliana ecotype Columbia was used in all experiments and is referred to as wild-type. For sterilisation of seed, packets were placed in a bell-container and exposed for three hr to chlorine gas generated by mixing 100 mL of commercial bleach with 3 mL of concentrated HCl. Seeds were placed either on Agar plates containing *Arabidopsis* growth media (Table 2.1) without sucrose, or on metro-mix soil, and stratified for 48 hr at 4°C in the dark. Plants were grown either under long-day conditions (16hr light/8 hr dark @ 150µmol/m^2/sec at 22°C), or under short-day conditions (8hr light/16hr dark @ 150µmol/m^2/sec at 22°C).

At growth media:	[Final]	
KNO_3	5	mM
KH_2PO_4	2.5	mM
$MgSO_4$	2	mM
$Ca(NO)_3$	2	mM
Fe(EDTA)	5	µM
H_3BO_3	70	µM
$MnCl_2$	14	µM
$CuS0_4$	0.5	µM
$ZnSO_4$	1	µM
$NaMoO_4$	0.2	µM
NaCl	10	µM
$CoCl_2$	0.01	µM

pH 5.8 with KOH, 7 g/L agar

Table 2.1 Concentrations of *Arabidopsis* growth media components

2.3 *Arabidopsis* DNA extractions

Purified DNA for cloning of constructs was extracted using a CTAB method modified from (Lodhi et al., 1994). The method was carried out as described except the 50% 5M NaCl used for precipitation was replaced by using an equal volume of isopropanol. The DNA was also resuspended in water rather than TE (10mM Tris-HCL pH 8.0, 1mM EDTA). For routine DNA extractions where genotyping was required, Edwards preperations were used (Edwards et al., 1991).

2.4 PCR genotyping

PCR was carried out using Platinum Taq (Invitrogen, Carlsbad, CA) in 20 µL reaction volumes, or 50 µL if PCR products were to be purified and sequenced. 2 µL of Edwards prep purified genomic DNA was used for each PCR, with final primer concentrations at 0.2 µM. PCR conditions were one cycle of 94°C/ 2 min; 30 cycles of 95°C/30 sec, 60°C/30 sec, 72°C/1-2min/; 1 cycle of 72°C for 5 min. 10 µL of each PCR reaction was run on a 1-1.5% TAE or TBE agarose gels. For sequencing, 40µL

of each PCR reaction was purified using a PCR purification kit (Qiagen, Valencia CA). Sequencing was carried out using ABI PRISM Big Dye Terminator v3.1 mix (Applied Biosystems, Foster City CA) using the recommended cycling conditions. The sequencing products were ethanol precipitated and analysed at John Curtin School of Medical Research, Australian National University, Canberra.

2.5 Competent *E.coli* cells and transformations

Competant cells of *XL10-gold* (Stratagene) and DH-5α (Hanahan, 1983) were prepared as described by Inoue et al., (1990), and transformed by heat shock as described in Sambrook et al., (1989). *TOP-10* competent cells used for gateway cloning were purchased from Invitrogen.

2.6 Gateway cloning

Genomic fragments were amplified using primers that had additional *att1* recombination sequences to allow integration into the pDONOR/Zeo entry vector using the BP recombination reaction (Invitrogen). PCR was carried out using high fidelity Phusion Taq polymerase (Finnzymes), with the following cycling conditions: 1 cycle of 98°C/ 30 seconds, 30 cycles of 98°C/10 seconds, 68°C/10 seconds, 72°C for 30s/kb-extension times varied according to template size, and 1 cycle of 72° for 10 min. Products were gel purified using a Wizard purification kit (Promega) and recombined with the pDONOR/ZEO entry vector in BP reactions. Recombinants were transformed into *E.coli* and grown on Zeomycin selection on low salt (85 mM NaCl) Luria Broth (LSLB) plates. Positive clones were grown overnight in liquid LSLB and plasmid purified using Qiaprep mini-preps. Plasmids were screened by restriction digest and sequenced to verify integrity of the cloned sequence. Positive clones were

recombined into destination vectors (pMDC99 for genomic constructs, pMDC164 for GUS constructs, pMDC32 for the *35S:MIR159c* construct) (Curtis and Grossniklaus, 2003), by LR recombination carried out overnight. Recombinants were transformed into *E.coli* and screened by restriction digestion. Positive clones were used for transformation of *Agrobacterium*.

2.7 Mutagenesis

For construction of *mMYB101*, a mutagenesis approach based on Zheng at al., (2004) was used. Forward and reverse primers were homologous for most of their length but overlapped by 13 bp and 14 bp at the 5' and 3' ends on either side of the *MYB101* miR159 target site. This was to minimise primer dimerization and maximise primer-template annealing. Furthermore the mutation was made to produce a novel *XhoI* restriction site in the *mMYB101* sequence to facilitate screening of mutant plasmids. Mutation was performed on *MYB101* genomic entry clones by PCR with Phusion Taq Polymerase. After digestion with *DpnI*, plasmids were transformed into *DH5a* and positive clones were screened by restriction digestion. Specific mutation of all 8 bases was verified by sequencing the *mMYB101* entry clone.

2.8 Transformation of *Arabidopsis*

Plasmids were electroporated into *Agrobacterium tumafaciens* GV3101 cells (Hellens et al., 2000), and plated on 50 µg/ml Rifamycin, 25 µg/ml Gentamicin, plus the appropriate plasmid selection. The plates were incubated for 48 hr at 28°C in the dark. Afterwards a single clone was plated out on the same selection for 48 hr. A streak of bacteria was then inoculated in a 10 mL liquid LB starter culture containing the same selection regime. After growth at 28°C overnight with shaking at 200 rpm, 250 µL of

this culture was used to inoculate a 250 mL culture of liquid LB with antibiotic selection only for the plasmid. The remaining culture was spun down for *Agrobacterium* plasmid preparation using Qiagen plasmid mini-preps. The protocol for this was identical to the manufacturers, except the volumes of re-suspension, lysis and neutralisation buffers were increased by 50%. Purified *Agrobacterium* plasmid was transformed into *E.coli XL-10* gold cells, then plasmid purified for verification that the plasmid was correct by restriction digestion. The 250 mL *Agrobacterium* culture was spun down and resuspended in 250 mL of milliQ water with 5% sucrose and 0.03% Silwet reagent (Clough and Bent, 1998). Pots containing 50-100 *Arabidopsis* plants at flowering were dipped in the media and wrapped in plastic for 2 days. Plants were then grown to seed set, and seeds were harvested. Seeds were sterilised as described and placed on selective media to identify primary transformants.

2.9 RNA extractions

RNA was extracted using a modified CTAB method (Chang 1993) from three day old imbibed seed, rosettes, siliques, flowers and mature plants. The method was as described with the following modifications: (1) Spermidine was not used in the extraction buffer (2) A phenol/chloroform/IAA extraction was carried out after adding SSTE (1M NaCl, 0.5% SDS, 10mM Tris-HCL pH 8.0, 1mM EDTA pH 8.0) to the RNA pellet, before the final chloroform:IAA extraction. For purification of small RNA, an equal volume of isopropanol was added to the LiCl precipitated lower phase, incubated at -20°C for two hr, and spun down (see Fig 2.1). The small RNA fraction pellet was washed twice with 70% ethanol.

Trizol (Invitrogen) was used for RNA extractions of inflorescences in quantitative stem-loop real time PCR (qSL-PCR), and for rosettes of *MYB101* transgenic plants. The procedure was as described by the manufacturer with the following modifications: (1) Approximately 500 mg of plant material and 1 mL of Trizol was used per extraction (2) homogenization was done using a mortar and pestle (3) The chloroform extraction was repeated. (4) Precipitation of RNA was carried out overnight at -20°C to maximise recovery of small RNA (5) Samples were heated only to 37°C after dissolving in nuclease free water.

All RNA concentrations were measured using a nanodrop spectrophotometer. For stem-loop qRT-PCR, where 10 ng/µl concentrations were required, serial dilutions with successive concentration readings were used. RNA was run on denaturing agarose gels to check integrity. Gels consisted of FA gel buffer (see below) with 1 % agarose, and 1.8 % 12.3 M formaldehyde. RNA was loaded in 5x loading buffer (see below) and ran in FA gel running buffer.

FA gel buffer
20 mM MOPS,
5 mM sodium acetate,
1mM EDTA
pH 7.0

FA gel running buffer
100ml 10x FA gel buffer
20 ml 37 % (12.3 M) formaldehyde
880ml nuclease free water

5x RNA loading buffer
16uL saturated bromophenol blue
80uL 500mM EDTA pH 8.0
720 uL 37 % (12.3M) formaldehyde
2 ml 100 % glycerol
3.084 ml formamide
4ml 10x FA gel buffer
Nuclease free water to 10 mL

2.10 DNAse teatment:

RQ1 DNAse (Promega, Madison WI) was used to treat RNA except for qSL-PCR (see below), where no DNase treatment was carried out. 100 µg was digested in 400 µL reactions consisting of 40 µL of 10x RQ1 buffer, 50 µL of RQ1 DNAse, and 10 µL of RNAsin (Promega) with nuclease free water to 400 µL. Digestions were carried out for 30 min at 37°C. RNA was then cleaned using Plant RNAeasy columns (Qiagen).

2.11 cDNA synthesis

cDNA synthesis was carried out using Superscript III reverse transcriptase (Invitrogen) according to the manufacturers protocol with an oligo dT primer (Invitrogen). For each RNA sample, three separate cDNA synthesis reactions were carried out using 5 µg of total RNA. The 20 µL cDNA reactions were diluted 50X in nuclease free water. This was to allow 10 µL of cDNA to be added in each 20 µL qRT-PCR reaction (see below) to reduce the error associated with pipetting smaller (1 µL) volumes.

2.12 Quantitative real-time PCR

qRT-PCR was carried out on Corbett rotor gene real-time PCR machines (Corbett Research, Sydney, Australia). Either a SYBR green jump start qRT-PCR kit (Sigma, St Louis, MO), or Platinum Taq (Invitrogen) with SYBR green (Sigma) added were used for the reaction chemistries. 10 µL of each cDNA was added to 9.6 µL of SYBR/Taq master mix and 0.4 µL of forward and reverse primers at 10 µmol each, for a final reaction volume of 20 µL. All qPCR reactions (for both reference and specific genes) were carried out in triplicate under the following cycling conditions: 1

cycle of 95°C/5 min, 45 cycles of 95°C/15 sec, 60°C/15 sec, 72°C/20 sec. Fluorescence was acquired at the 72°C step. A 55°C to 99°C melting cycle was then carried out. *Cyclophillin* (At2g29960) was used to normalise gene expression using the comparative quantitation program in the rotor gene software package (Corbett). The values for each set of three cDNAs (see above), representing the average of triplicate assays, were averaged, allowing for a calculation of the standard error of the mean (SEM).

2.13 Northern analysis of mature miR159a expression in seeds.

20 µg of the small RNA fraction from three-day imbibed seed total RNA was separated on a 17% polyacrylamide gel. The gel was made in a 50 mL volume and consisted of 21 g of urea in 21 mL of 38% polyacrylamide (acrylamide:bis, 37.5:1), 5 mL of 10x TBE and 8.85 mL of sterile distilled water, warmed to 50°C. Afterwards, 25 µL of TEMED and 300 µL of APS was added and mixed quickly before pouring the gel to polymerise for at least one hour. After adding 1x TBE as running buffer, urea was rinsed from the sample wells using a hamilton syringe, and the gel pre-run for at 180 V for ~ 1 hour. The RNA samples were denatured in formamide at 65°C for 5 min and RNA loading buffer (see above) before adding to the wells. The gel was run at 60 V for 6 hr. The gel was electro blotted to Hybond XL (Amersham, NJ) and UV cross-linked. The blot was pre-hybridised and hybridised overnight at 40°C in 125 mM Na_2PO_4 (pH 7.2), 250 mM NaCl, 7% SDS and 50% formamide. [γ^{32}P]UTP labelled miR159a and U6 probes were a gift from Andrew Eamens at CSIRO Plant Industry. Membranes were washed twice with 2x SSC and 0.2% SDS for 30 min at 50°C, and imaged using a Fujifilm FLA-5000 phosphorimager.

2.14 Quantitaive Stem-loop qRT-PCR miRNA analysis.

For TaqMan stem-loop qRT-PCR miRNA analysis (qSL-PCR), RNA was prepared using Trizol as described above. For the assays, Applied Biosystems assays were used, and the manufacturers instructions were followed with the following modifications: for each RNA sample, there were three stem-loop cDNAs made, and the reverse transcriptase (RT) step was multiplexed using both *sno101* RT primer and miR159a, miR159b or miR159c primer. Additionally, the cDNA (15 µL) was diluted with 86.4 µL of nuclease free water, so that 9 µL of RT reaction could be pipetted into 20 µL total PCR reaction volume, to reduce the error associated with pipetting small (1 µL) volumes. Each cDNA was assayed in triplicate on a Corbett real-time PCR machine. Expression of miR159 was normalised to *sno101*, using the comparative concentration analysis program of rotor gene software (Corbett).

2.15 Modified 5'- Rapid Amplification of cDNA Ends (RACE) of cleaved miR159 targets

mRNA was purified from the same inflorescence RNA samples used for qRT-PCR analysis of miR159 targets in wild-type and *mir159ab*. An Oligotex mRNA purification kit was used (Qiagen) with 100 µg of starting total RNA. A Gene-racer kit (Invitrogen) was used for 5'- RACE, except the de-capping protocol was not carried out, and the adapter was ligated directly to mRNA. PCR of *MYB33* and *MYB101* sequence downstream of the miR159 cleavage site was used as a control to check 5' cDNA amplification was successful. The products from the second (nested) round of 5' RACE were gel purified using a Wizard preps PCR purification kit (Promega) and ligated into pGEM-T easy (Promega). Plasmids were transformed into *E.coli* XL-10 gold cells and selected on ampicillin plates containing 100 µL of

100mM IPTG and 20 µL of 50 mg/ml X-Gal. Individual colonies were grown overnight in LB with 50 µg/ml ampicillin and purified using Qiagen mini-preps. Clones were digested with *NotI* to verify they contained inserts of the correct size, and were sequenced.

2.16 Histochemical Analysis of B-glucuronidase (GUS) activity.

In situ GUS activity staining was performed using the method of Jefferson (Jefferson et al., 1987). The staining reagent consisted of 100 mM Na Phosphate buffer, Ph 7.0, 10 mM EDTA, 0.1% triton X-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. Tissues were transferred to this reagent in 1.5 mL Eppenedorf tubes, vacuum infiltrated for 2 min, and left overnight or as described elsewhere at 37°C. Afterwards, stained tissues were rinsed three times using 70% ethanol.

2.17 Preparation of GUS stained anther sections for light microscopy.

Inflorescences were stained as in GUS reagent for 48 hr at 37°C and dehydrated in a graded ethanol series (70%, 95%, 100%). Inflorescences were then infiltrated and embedded with LR white resin (London Resin Company). 2 µm transverse sections were made with a Leica Ultracut 6 ultramicrotome (Leica UK Ltd, Milton Keynes, UK). Sections were stained with 1% toludine blue for 1 minute.

2.18 Images

Digital photographs of rosettes, siliques and whole plants were taken at the CSIRO Phytotron studio, Canberra, Australia. Scanning electron microscopy of stamens, anthers, pollen and seed was performed by gold-coating tissues using a high

resolution splutter coater (Bio-Rad, Hercules CA), and examined with a Cambridge S360 SEM (Cambridge, UK). Images of GUS stained anther sections, three-day-old seedlings, roots and root hairs were taken with a Leica DMR upright microscope for bright-field and dark-field microscopy. Images of five and ten day old GUS stained seedlings, inflorescences, grouped seeds, and individual flowers were taken with a Leica MZFLIII dissecting microscope.

Chapter 3

Characterisation of *Arabidopsis mir159* T-DNA mutants

If something is redundant
it no longer has a function

-Tim Begbie (Senior Executive Lawyer)

3.1 Introduction

miR159 is among the most ancient miRNAs in the plant kingdom, with an evolutionary history extending back to Lycopods (Axtell and Bowman, 2008). In *Arabidopsis*, deep sequencing of small RNA populations has revealed miR159 to be one of the most abundant miRNAs (Kasschau et al., 2007; Rajagopalan et al., 2006). Despite this long evolutionary history and strong expression, the importance of miR159 in plant development remains unclear.

A loss-of-function approach is conceptually a powerful means of understanding gene function. Yet redundancy often presents problems where multiple gene family members exist. Accordingly it has been suggested that in addition to their small size, redundancy has limited discovery of miRNA loss-of-function mutants, as they are often encoded by multigene families.

In *Arabidopsis*, loss-of-function mutants with phenotypic consequences have only been reported for the miR164 family (Nikovics et al., 2006; Sieber et al., 2007). In part due to the paucity of knockouts available, roles for many miRNAs have been ascribed based on bioinformatics and/or overexpression studies (Schwab et al., 2005). However there are obvious limitations to these methods, where bioinformatic approaches cannot account for the complexity of factors that affect miRNA function. For overexpression studies, artificial spatial and temporal transgene expression may distort the true function of a particular miRNA. Further inferences of miRNA roles have been made using miRNA resistant targets and assessing the developmental consequences. Though sometimes informative, such

studies are limited by their narrow scope to encompass all potential targets of a particular miRNA, and again may be complicated by artifactual transgenic effects.

The *Arabidopsis* miR159 family is an obvious candidate for investigating the role of miRNAs. Firstly it is highly conserved in plants and may represent similar function across the plant kingdom. Secondly it is highly expressed which suggests a noticeable impact on gene regulation. Finally it only contains three members, which makes it an ideal candidate for characterisation using loss-of-function approaches. This chapter describes the molecular and phenotypical characterisation of T-DNA insertional mutants of *Arabidopsis MIR159* genes.

The main aims of this chapter are:

(1) Determine the physical structure of the T-DNA mutant loci for all three *MIR159* genes

(2) Phenotypical characterisation of single, double and triple *mir159* mutants

(3) Examine the tissue expression patterns of *MIR159a* and *MIR159b*,

(4) Measure the levels of precursor and mature miR159 in the various *mir159* T-DNA mutants.

3.2 Results

3.2.1 Sequence similarities of *MIR159* genes

There are three miR159 members in *Arabidopsis* (Palatnik et al., 2003). *MIR159a* and *MIR159b* are on chromosome I and *MIR159c* is on chromosome II (Jones-Rhoades 2004). The stem-loop regions (pre-miRNAs) of the *MIR159a* and *MIR159b* genes share strong homology as shown by using pairwise blast (Altschul et al., 1997) (Fig. 3.1A). A phylogenetic tree (Larkin et al., 2007) was created using all known rice and *Arabidopsis MIR159a* stem-loop sequences (Griffiths-Jones et al., 2008), and the *Arabidopsis MIR159a* and *MIR159b* stem-loops branch together strongly (Fig. 3.1 B). The close similarities between *MIR159a* and *MIR159b* suggested they may be a redundant gene pair, whilst *MIR159c* appears to be more distantly related.

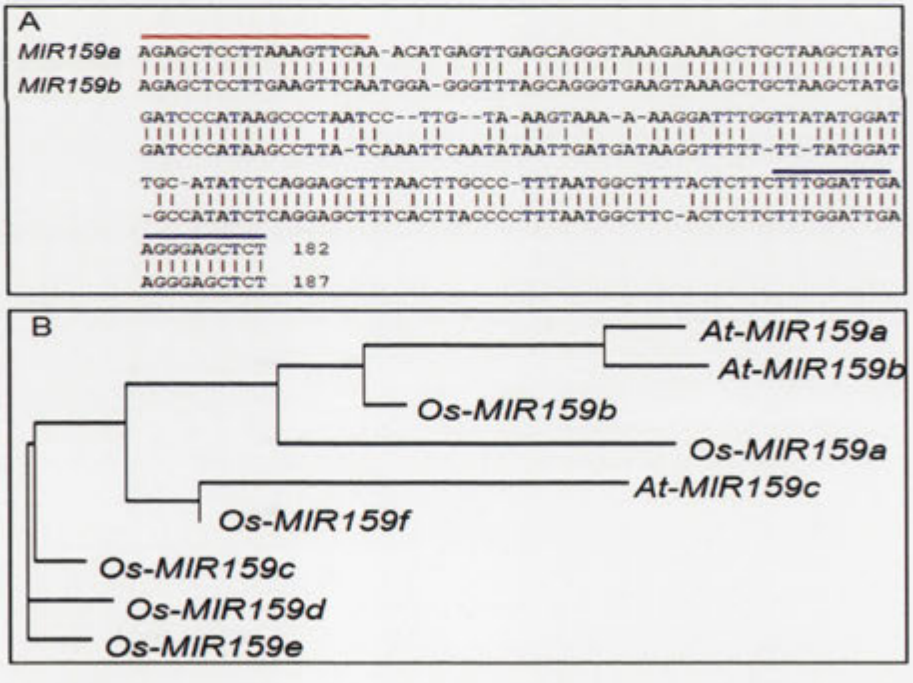


Fig 3.1 Pair-wise comparison of *MIR159a* and *MIR159b* stem-loop sequences and relationship of *Arabidopsis* and *rice MIR159* stem-loops.
(A) *MIR159a* and *MIR159b* stem-loops (pre-miR159) were compared using pairwise blast. Red and blue bars indicate location of miRNA* and miRNA sequences respectively.
(B) A Cladogram tree based on *MIR159* stem-loop regions from *Arabidopsis* and rice

3.2.2 Genomic context of the *Arabidopsis MIR159* genes

According to TAIR (www.arabidopsis.org), all three *MIR159* genes reside in intergenic regions of *Arabidopsis* (Fig 3.2). The longest primary transcripts as determined by 5' and 3' RACE are 806 bp for *MIR159a* and 900 bp for *MIR159b* (Data provided by Frank Gubler, Allen et al., 2007). The transcription start and stop site for *MIR159c* has not been determined. Both *MIR159b* and *MIR159c* have transposable elements located in their putative promoter regions, however for *MIR159c* this is only 214 bp upstream of the stem-loop region, which would suggest that it may have a major impact on the expression of this gene.

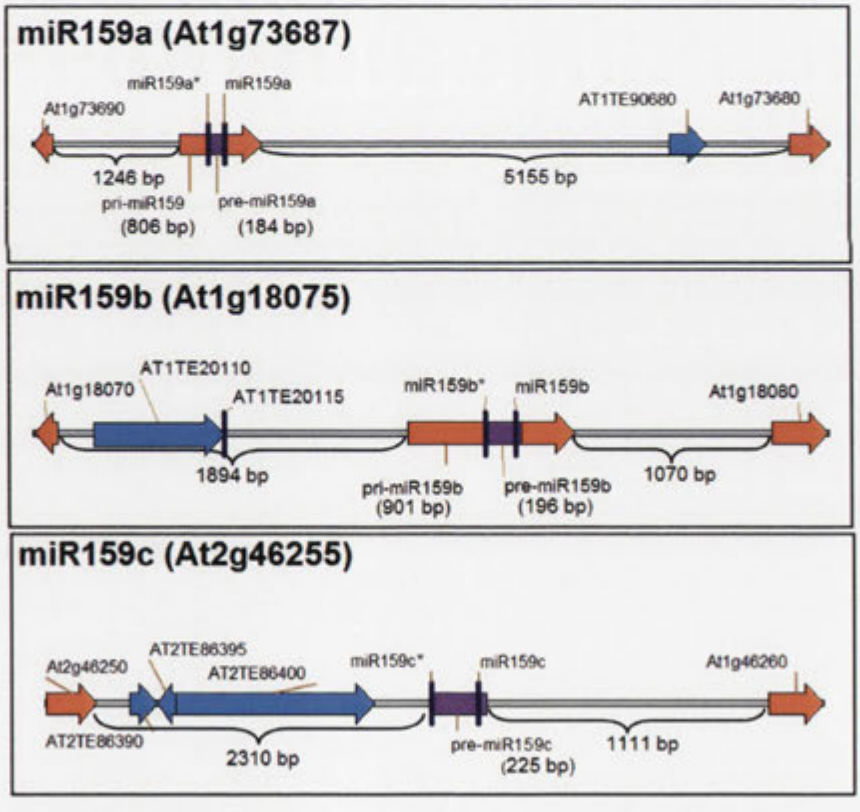


Fig 3.2
Genomic context of *Arabidopsis MIR159* genes
Intergenic regions in which pri, pre (stem-loop) and mature miR159 reside are shown to scale, with segments of adjacent upstream and downstream genes indicated. No pri-miRNA is indicated for miR159c as it has not been determined. Transposable elements (shown by blue arrows or lines) in each region are shown to scale.

3.2.3 T-DNA mutations in *MIR159* genes affect pri-miRNA levels

To determine the role of miR159 in *Arabidopsis* a loss-of-function strategy was used. The SIGnAL database (Alonso et al., 2003) describes T-DNA mutations in all three *MIR159* genes. These are SAIL 430 F11 (*mir159a-1*), GABI 468 E06 (*mir159a-2*), SAIL 770 G05, (*mir159b*) and SAIL 248 G11 (*mir159c*). PCR and sequencing using T-DNA vector and gene specific primers was used to determine the precise location of each T-DNA insertion. For all mutants, it was found that insertions were inverted duplications, which is relatively common for T-DNA insertions in plants (Jorgenson et al., 1987).

For *mir159a-1*, the T-DNA had integrated at a position 232 bp upstream of the region predicted to form the stem-loop (Fig. 3.3), but downstream of the mapped transcription start site. Using qRT-PCR, expression of *pri-mir159a-1* was reduced more than six-fold but not eliminated, raising the possibility that *mir159a-1* was a hypomorphic mutation.

In an effort to find a *mir159a* mutant containing an insertion within the stem-loop region that would possibly lead to complete loss of miR159a, the SIGnAL database was searched and a second mutant that contained T-DNA potentially within the *MIR159a* stem-loop region was found. However PCR and sequencing determined this second allele (*mir159a-2,* Fig. 3.3) contained the T-DNA insertion 3 bp downstream of the predicted stem-loop structure. qRT-PCR measurement of *pri-mir159a-2* transcript showed a two-fold increase relative to wild-type, suggesting the T-DNA is influencing transcription and/or processing of *MIR159a*. Given that transcript had not been eliminated and the insertion was outside the stem-loop region, it was possible that *mir159a-2* was also a hypomorphic mutation.

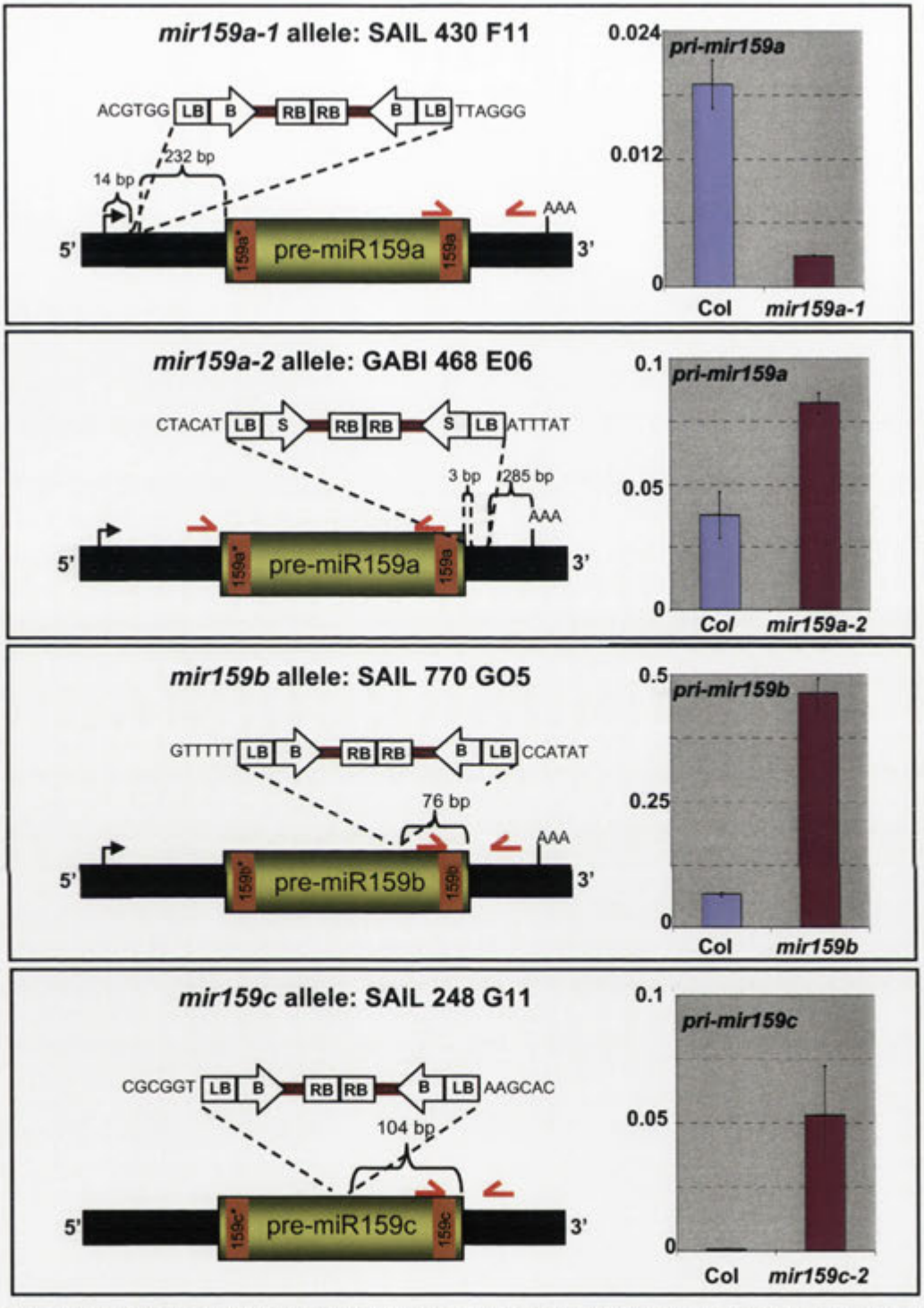


Fig 3.3 Structure of T-DNA insertions and effect on pri-miRNA levels for *mir159* mutants. For *mir159* mutants, the position of T-DNA insertions and their proximity to the pre-miRNA stem-loop (indicated by yellow box) is shown within the context of genomic flanking sequence for each mutant locus (not to scale). For *mir159a* mutants, where T-DNA insertions lie outside the stem-loop, the distance to the start of transcription (indicated by an arrow), or the 3' end of the longest transcript as determined by RACE (indicated by AAA) is also indicated. Mature miRNA and miRNA* sequences are indicated. Red arrows represent primer regions used for qRT-PCR analysis of pri-miR159. LB=Left Border, RB=Right Border, B= Basta resistance gene, S=Sulfadiazine resistance gene. Corresponding levels of *pri-miRNA* transcript for each mutant line were determined by qRT-PCR. Values represent RNA levels relative to cyclophillin. Error bars represent standard error of the mean (SEM).

For *mir159b*, the T-DNA had integrated within the stem-loop region, at a position 76 bp upstream from the 3' end of the *mir159b* stem-loop (Fig. 3.3). qRT-PCR measurement showed *pri-mir159b* transcript in *mir159b* was elevated more than seven-fold relative to wild-type, suggesting it is not able to be properly processed. Given that the T-DNA had integrated between the miR and miR* sequences, it is most likely their separation by several kb of T-DNA would completely disrupt the ability of this mutant locus to make a transcript with the secondary structure required to form mature miR159b. Therefore this mutant is likely a null allele of *MIR159b*.

For *mir159c*, the T-DNA had integrated within the stem-loop region, at a position 104 bp upstream from the 3' end of the miR159c stem-loop (Fig. 3.3). Endogenous levels of *MIR159c* transcript were extremely low, which corresponds to similarly low levels of mature miR159c from deep sequencing of *Arabidopsis* (Backman et al., 2008; Fahlgren et al., 2007). The presence of a large transposable element only 214 bp upstream of the 5' end of the miR159c stem-loop (Fig. 2) may be responsible for low levels of *MIR159c* transcript. However elevated *pri-mir159c* transcript could be detected in *mir159c* mutants, suggesting that the T-DNA is affecting transcription and/or processing of this gene. Given the location of the T-DNA between the miR and miR* sequences, any transcript from this allele would be unlikely to form the secondary structured required to process mature miR159c. Therefore this is most likely a null allele of *MIR159c*.

3.2.4 Phenotypes of *mir159* mutants

3.2.4.1 A *mir159a-1/mir159b* double mutant displays pleiotropic developmental defects

All *mir159* mutants were examined to determine if the alterations seen in *pri-mir159* levels led to phenotypic changes. The morphological phenotypes of all *mir159* single mutants were indistinguishable from wild-type (data not shown). However, the sequence similarities between *MIR159a* and *MIR159b* suggested functional redundancy. This prediction was confirmed by a homozygous double *mir159a-1/mir159b* mutant (provided by Tony Millar), hereafter called *mir159ab;* this mutant displayed pleiotropic developmental phenotypes, reflecting functional redundancy for this gene pair (Fig 3.4 A-D). Compared to wild-type, *mir159ab* plants were stunted, showing smaller stature. Rosettes had distinctive upward curling leaves. Mature siliques were shorter than wild-type, indicating reduced fertility and seed set. Seeds were generally reduced in size with irregular shapes compared to wild-type. The only tissue examined that appeared normal was pollen; SEM examination revealed that pollen grains were of identical shape and size to wild-type (Fig. 3.4 E-F).

3.2.4.2 *mir159a-2/mir159b* double mutants appear indistinguishable from wild-type.

A cross was made between *mir159a-2* and *mir159b*. Homozygous *mir159a-2/mir159b* mutants were confirmed by PCR and showed no obvious phenotypic differences compared to wild-type (Fig 3.4 G), suggesting that despite the presence of a T-DNA insertion only 3 bp downstream of the stem-loop region, there remains sufficient miR159 in this mutant to fulfill function.

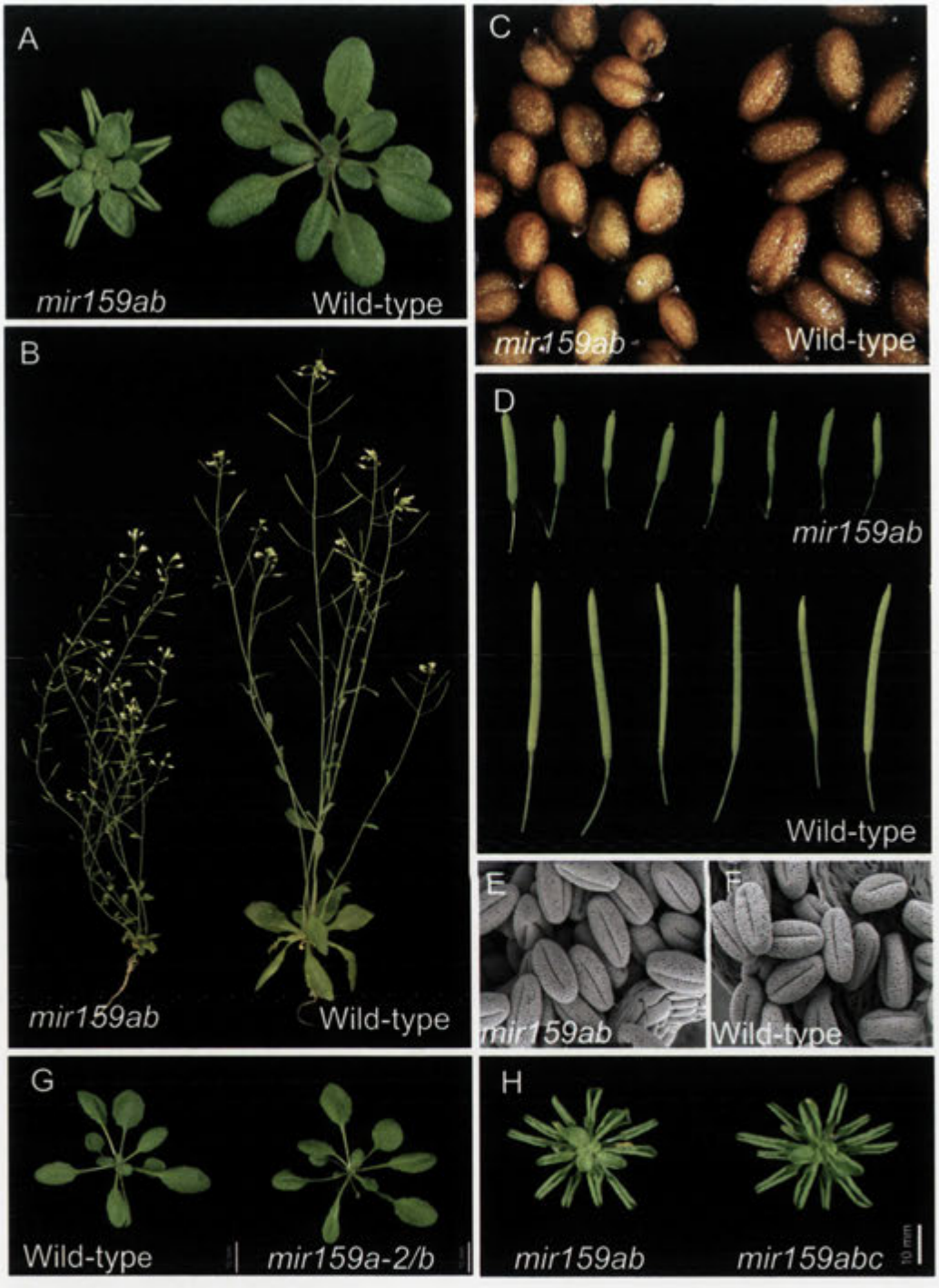


Fig 3.4 Phenotypes of *mir159* mutants

The phenotype of the *miR159ab* mutant is pleiotropic, with a range of developmental defects including: upwardly curled leaves (A) smaller stature (B), smaller seeds (C) and siliques (D). In contrast pollen was normal in *mir159ab* (E) compared to wild-type (F) . Aerial view of rosettes comparing wild-type with *mir159a-2/b* (G) and *mir159ab* with *mir159abc* (H).

3.2.4.3 *mir159a-1/mir159b/mir159c* triple mutants appear indistinguishable from *mir159ab*

Finally a cross was made between *mir159ab* and *mir159c*. No phenotypes additional to those of the *mir159ab* mutant were observed in PCR confirmed homozygous *mir159a-1/mir159b/mir159c* (*mir159abc*) mutants (Fig 3.4 H). Additionally no phenotypes were seen in homozygous *mir159a-1/mir159c* or *mir159b/mir159c* mutants (data not shown). These results suggest there is no additional redundancy between miR159a/b and miR159c. The lack of additional phenotypes seen in *mir159abc* when compared to *mir159ab*, combined with deep sequencing data that shows miR159a and miR159b comprise over 99.9% of miR159 levels, argues that miR159a and miR159b are the predominant members of this family in terms of abundance and functional importance. However the possibility that miR159c may play a less obvious role will be investigated in chapter 6.

3.2.5 *MIR159a* and *MIR159b* have overlapping expression patterns.

Expression of promoter:GUS fusion constructs for *MIR159a* and *MIR159b* in *Arabidopsis* was examined to determine if the redundancy evidenced by the *mir159ab* phenotype was reflected in similar expression patterns for these genes. Transgenic lines were provided that had been transformed with constructs containing sequences immediately upstream of the *MIR159* stem-loop regions extending to the next gene (approximately 1.7 kb for *MIR159a* and 2.4kb for *MIR159b*) fused to GUS (Allen et al., 2007). Multiple lines were grown and examined for GUS expression. Examination after 18 hr of GUS staining showed strong expression for both constructs throughout the plant; strong staining was observed in 48 hr old seedlings (Fig. 3.5 A, D), root tips (Fig. 3.5 B, E) secondary roots (Fig. 3.5 G, H) and

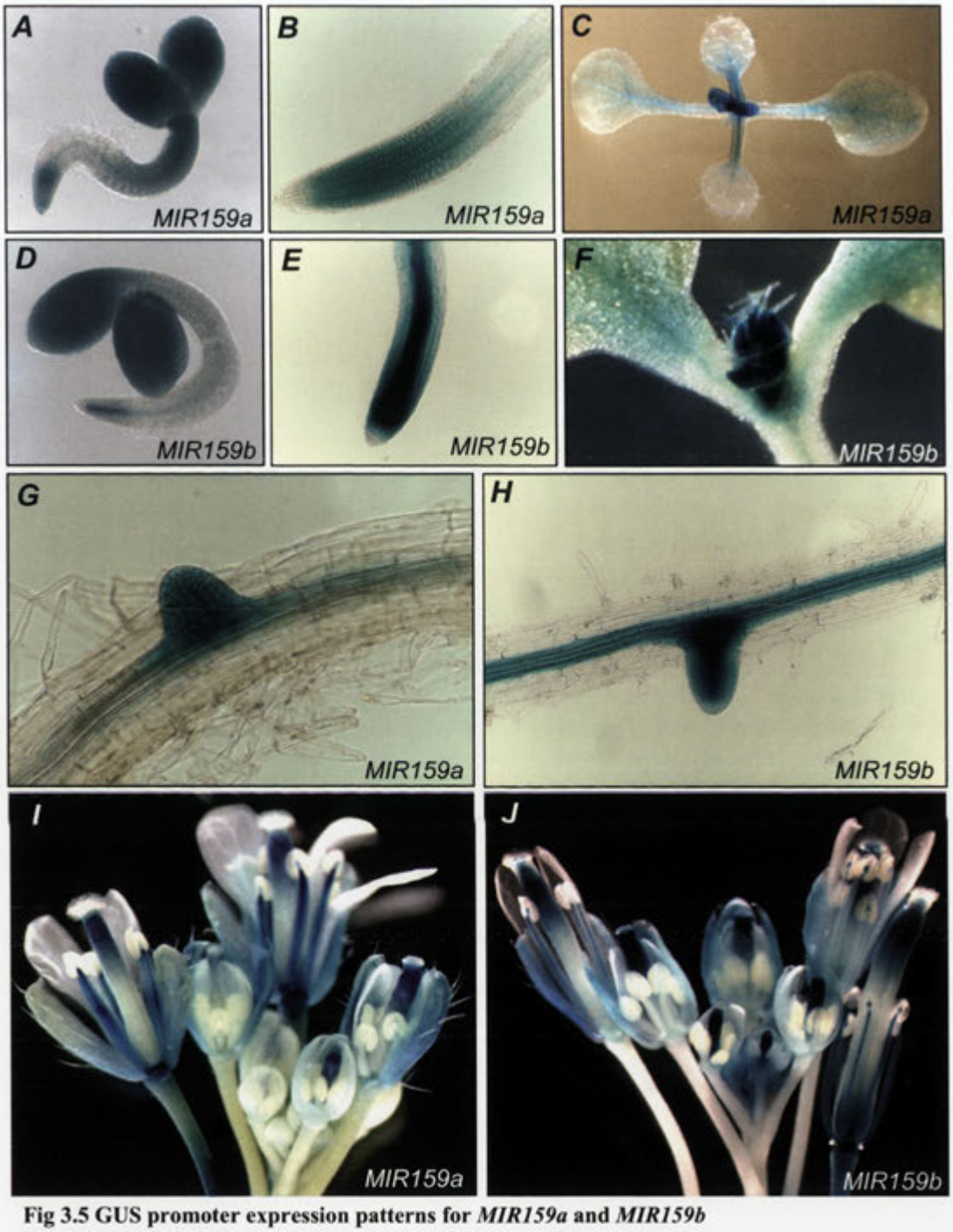


Fig 3.5 GUS promoter expression patterns for *MIR159a* and *MIR159b*
Expression of a *MIR159a* and *MIR159b* overlaps, as indicated by GUS reporter constructs. Examples of overlapping expression are shown for two day old seedlings, roots tips and shoot apical regions respectively for *MIR159a* (A,B,C) and *MIR159b* (D,E,F), as well as secondary roots and flowers for *MIR159a* (G,I) and *MIR159b* (H,J). (Images C,F, I,J provided by Anthony Millar)

the shoot apex region (Fig. 3.5 C, F). Staining was also observed in the inflorescence; in receptacles, anther filaments, sepals and carpels (Fig 3.5 I, J). Conspicuously, GUS staining could not be seen in anthers, consistent with morpologically wild-type looking pollen observed in *mir159ab.* From the examination of promoter activity using the GUS reporter gene, it could be concluded that both genes share high similarities in the pattern of their expression, which is consistent with the functional redundancy evidenced in the *mir159ab* mutant.

3.2.6 Levels of miR159 in *mir159ab* are dramatically reduced

The phenotype of the *mir159ab* mutant together with corresponding changes to pri-miRNA levels, suggested the *mir159a-1* and *mir159b* T-DNA mutations were affecting the production of mature miR159. To determine the effect of the T-DNA mutations on mature miR159 levels in *mir159ab*, northern blotting was used. RNA was extracted from three-day old imbibed seed as pri-miR159 precursor was previously shown to be strongly expressed at this stage (Allen et al., 2007), and the small RNA fraction purified. After hybridization with a miR159a probe (Fig 3.6), a band could be detected in wild-type. A faint band could be detected in *mir159a*, and a stronger band in *mir159b*, likely representing cross-hybridization of the probe to miR159b in *mir159a*, and to miR159a in *mir159b*. No miR159 could be detected for the *mir159ab* mutant, showing miR159 had been eliminated to at least below the detection limits of northern blotting.

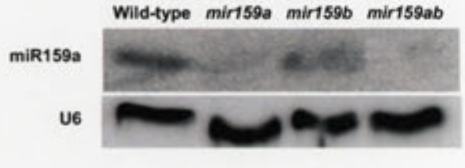


Fig 3.6 Detection of miR159 in *Arabidopsis* imbibed seed.
sRNA isolated from three-day old imbibed seed of wild-type, *mir159a*, *mir159b* and *mir159ab* was probed with a ^{32}P-UTP miR159a oligonuceotide. It was then stripped and re-probed with a ^{32}P-UTP U6 oligonulceotide.

3.2.7 Stem-loop qRT-PCR analysis of *mir159* T-DNA mutants

3.2.7.1 qSL-PCR assays discriminate closely related miR159 family members.

In an effort to more precisely determine the affect of the miR159 T-DNA mutations on mature miR159 accumulation, a recently developed ABI TaqMan Stem-loop PCR method was employed (referred to hereafter as qSL-PCR). In the case of miR159, the specificity of the assay can only be conferred at the reverse transcriptase (RT) reaction step, where the three different RT primers contain a six nucleotide region specific to each individual miR159 member (Fig. 3.7). All three miR159s are identical apart from the final two nucleotides at their 3' end, where miR159a and miR159b differ by one base, and miR159c differs by one base compared with miR159b and two bases compared to miR159a (Fig 3.7).

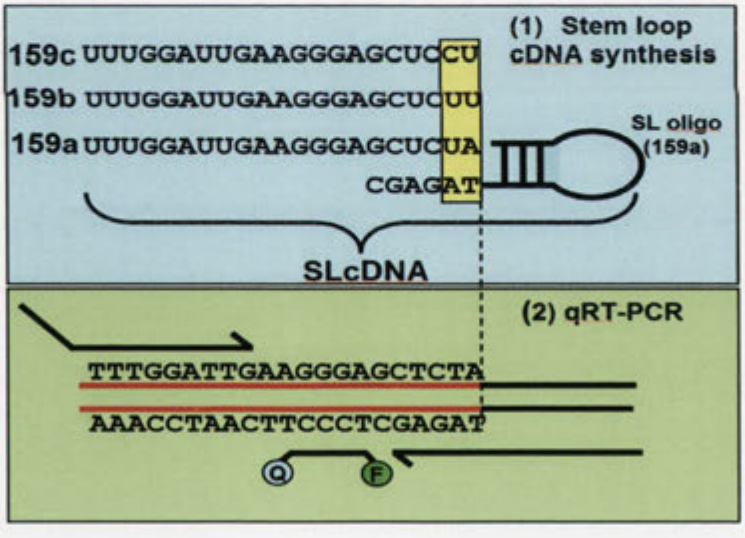
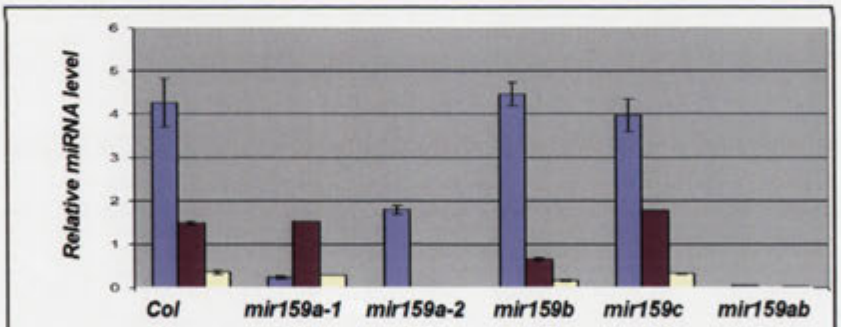


Fig 3.7: Use of qSL-PCR for miR159 detection in *Arabidopsis*.
The final six nucleotides at the 3' end of each stem-loop oligonucleotide contain sequences complementary to each miR159 member. In the example shown the miR159a stem-loop oligonucleotide contains sequence perfectly homologous to miR159a, but with mismatches to miR159b and miR159c (shown in yellow box). The differences between the miRNAs at this 3' end form the basis of the assays specificity, as miR159 sequences are identical elsewhere. Subsequent qPCR provides no further opportunity for discrimination of individual members as qPCR primers sequences will be identical (forward), or specific only to the stem-loop cDNA that was synthesized by each particular assay (reverse). The sequences and position of the TaqMan probe and primers are proprietary and therefore their position shown is arbitrary.

Deep sequencing of *Arabidopsis* inflorescences shows the relative abundance of miR159 in the *Arabidopsis* ecotype Columbia to be 87.2%, 12.6% and 0.2% for miR159a, b and c respectively (Backman et al., 2008). Therefore to initially test the specificity of the assays, a comparable *Arabidopsis* RNA sample was prepared. This sample was made from the same tissue (inflorescences) of the same ecotype (Columbia) grown using the same conditions (16 hour long-days). This will allow an approximate comparison of the qSL-PCR method with the deep sequencing data-set.

Using the qSL-PCR assays, the relative abundance of miR159a, b and c was 69.7%, 24.4% and 5.9% respectively (Fig 3.8). The trend in the relative abundance of the different members correlates with the deep sequencing data, although it would appear either biological variation or imperfect specificity of the assays has led to differences in the miR159 expression values between the two data sets.



Assay	Line: col	159a	159a-2	159b	159c	159ab
miR159a	4.26	0.24	1.79	4.46	3.98	0.06
miR159b	1.49	1.54	n.t	0.64	1.78	0.01
miR159c	0.36	0.29	n.t	0.16	0.32	0.04

Fig 3.8 Quantification of mature miR159a, miR159b and miR159c in the *mir159* mutants by qSL-PCR. Total RNA from inflorescences was subject to stem-loop qRT-PCR (qSL-PCR) and the level of each miR159 member relative to *sno101* was determined by comparative quantitation analysis. Numbers in the table correspond to the values on the graph above. Error bars represent SEM. n.t = not tested

3.2.7.2 Use of *mir159* mutantsreveals specificity of qSL-PCR assays.

To determine if the individual qSL-PCR assays were cross-reacting with different miR159 members, the *mir159* T-DNA single mutants were assayed by qSL-PCR, and compared with assays of the double *mir159ab* mutant, where mature miR159 levels are negligible. This analysis revealed the assays generally had high, but not absolute specificity. For example comparison of miR159a levels in *mir159a-1* (0.24) with *mir159ab* (0.06) indicates the miR159a assay is detecting miR159b or miR159c in *mir159a-1,* where these members are present and can potentially cross-react with the miR159a RT-primer. Similarly, the levels of miR159c found in wild-type (0.36) and *mir159c* (0.32) are likely representative of miR159c RT-primer cross-reaction with miR159a and miR159b, as demonstrated by the level of miR159c revealed in *mir159ab* (0.04) where negligible miR159a or miR159b is present.

3.2.7.3 qSL-PCR assays confirm miR159 is reduced to negligible levels in *mir159ab.*

qSL-PCR measurement of miR159 levels in the *mir159ab* mutant shows a reduction of miR159a and miR159b levels to approximately 1% of wild-type (Fig 3.8), which is reflective of the previous result (see 3.3.6 above) from seed RNA using northern blotting. This result is consistent with earlier analysis where only *mir159ab* plants showed a phenotype different to wild-type, as there is a drastic reduction in miR159 levels in this mutant compared to any of the single mutants. This also demonstrates that although *pri-mir159a-1* was reduced only six-fold, there has been a greater reduction in mature miR159a

in *mir159a-1*, implying the T-DNA mutation is not only reducing transcription of *mir159a-1* but considerably affecting processing of miR159a from this mutant transcript. Therefore this mutant could be considered a strong loss-of-function allele of *MIR159a*.

3.2.7.4 *mir159a-2* transcript can be processed despite an insertion 3 bp downstream of the stem-loop.

For the *miR159a-2* allele, according to the qSL-PCR, there has been a 58% reduction in miR159a despite the T-DNA insertion being only 3 bp downstream of the predicted stem-loop region. The absolute level of miR159a reduction in *mir159a-2* is likely to be less than indicated by the assay, due to cross-reaction of the miR159a RT primer with miR159b sequences giving an artificially higher miR159a level. Accordingly this modest impact (relative to *mir159a-1*) on total miR159 levels is reflected by the wild-type phenotype of homozygous *mir159a-2/mir159b* plants (Figure 3.4). In further contrast to *mir159a-1*, where the T-DNA has integrated 223 bp upstream of the stem-loop region, this result for *mir159a-2* shows that at least for miR159a, sequences extremely close to but downstream of the stem-loop region can tolerate disruptions with a lesser impact on miRNA production than upstream insertions.

3.3 Discussion

3.3.1 The *mir159ab* double mutant demonstrates functional redundancy.

Loss-of-function mutants in *MIRNA* genes were phenotypically characterised before it was realized that miRNAs, or any other regulatory sRNA pathways existed (Ambros and Horvitz, 1984). Yet there are still few examples of loss-of-function *MIRNA* gene mutants in both animals and plants. This scarcity has been thought due to their small size and/or potential redundancy, as most miRNAs are members of multi-gene families (Xie et al., 2004). Here it has been shown that only *mir159ab* displays a mutant phenotype, demonstrating that in accordance with the latter notion, miR159a and miR159b are fully redundant to each other. A corollary of this finding is that only a single copy of wild-type *MIR159a* or *MIR159b* can carry out miR159 function. Given this study found miR159a to comprise at least ~70% of total miR159 levels in *Arabidopsis*, it would be predicted that miR159 can be reduced to at least 15% in a *mir159a/MIR159b/mir159b* mutant, and still achieve its function, implying that miR159 is produced in substantial excess.

The notion that miR159a and miR159b are functionally redundant is supported by several additional lines of evidence. Firstly, this study found *MIR159a:GUS* and *MIR159b:GUS* reporter constructs produced highly similar expression patterns throughout different developmental stages. Secondly, *MIR159a* and *MIR159b* are highly similar at the sequence level, having complementary sequences within their stem-loop regions. Thirdly, *MIR159a* and *MIR159b* reside in a region of chromosome I that appears to have undergone a duplication event within the last 30 Myr (Maher et al., 2006). These are all hallmarks of

redundant genes, and although alone they are insufficient to establish that *MIR159a* and *MIR159b* are redundant, the mutant phenotype found only in homozygous *miR159ab* plants confirms this.

This raises the general question of what selective advantage is gained in maintaining two copies of a gene when apparently one can suffice. Redundancy manifested through gene duplication is common in nature (Louis, 2007). After duplication events, subsequent redundant genes may follow several fates, ranging from specialization to loss-of-function. Therefore redundancy offers not only a safeguard against losing functionality for a particular gene, but also provides a basis for genetic diversification. The results from this chapter cannot predict if *MIR159a* and *MIR159b* are taking any of these evolutionary paths. The fact that miR159 is produced in substantial excess may suggest a strong requirement to maintain functionality of miR159 by retaining a redundant gene pair. Alternately, it remains possible that selection has necessitated maintenance of both copies for particular roles that may be required under different conditions than those experienced in the controlled conditions of this study. Either way, the presence of *MIR159a* and *MIR159b* ensures a necessarily robust means of target repression in *Arabidopsis*, as failure to do this would have deleterious results.

3.3.2 Relative importance of the miR159 family members

The pleiotropic phenotype of *mir159ab* corresponds with the widespread pattern of GUS activity seen for *MIR159a* and *MIR159b* promoter constructs, and demonstrates that expression of miR159a and miR159b throughout *Arabidopsis* is important for normal

development. Conspicuously, anthers showed a lack of GUS activity, and pollen was the only tissue examined that appeared indistinguishable from wild-type. These observations support the notion that miR159 is absent or low in these tissues. This is consistent with a recent analysis of miRNAs in pollen, where miR159a and miR159b were among certain miRNAs not detected in this tissue (Chambers and Shuai, 2009).

Unlike *MIR159a* and *MIR159b*, T-DNA disruption of *MIR159c* does not appear to lead to any developmental consequence, where *mir159abc* mutants were indistinguishable from *mir159ab*, and *mir159c* plants were indistinguishable from wild-type, at least at the gross morphological level. The lack of an apparent *mir159c* phenotype is reflected by the scarcity of miR159c, as determined in this study by qSL-PCR (Fig 3.8), and previously shown by deep sequencing (Backman et al., 2008). A possible explanation for the low abundance of miR159c is suggested by an examination of sequences immediately upstream of miR159c; a large transposable element lies only 214 bp 5' upstream of the miR159c stem-loop region, and thus likely resides in the *MIR159c* promoter region or possibly the pri-miRNA itself. The evolutionary origins of *MIR159c* are unclear. *MIR159c* may have arisen independently of *MIR159a/b* and undergone selection for a more specialized and/or subtle role. These possibilities will be considered and examined in chapter 6.

3.3.3 Position of T-DNA relative to pre-miRNA has major impacts on gene activity.

Measurement of miR159 levels by qSL-PCR provided an explanation for why there was no phenotype observed in *mir159a-2/b*, in contrast to *mir159a-1/b* plants: the level of miR159a reduction in *mir159a-2* was considerably less than in *mir159a-1*. It is of interest

that a T-DNA insertional mutation close to but downstream of the stem-loop (only 3 bp for *mir159a-2*) has far less of an impact on miR159 accumulation than a similar T-DNA mutation (*mir159a-1*) that resides only 14 bp into the transcribed region and 232 bp upstream of the stem-loop region. This implies, at least for *MIR159a*, integrity of the native sequence upstream of the stem-loop is more critical than for downstream regions. Curiously, the transposable element directly upstream of the miR159c stem-loop lies at a similar distance as the upstream T-DNA in *mir159a-1*. Given that miR159c is virtually undetectable (like miR159a in *mir159a-1*), it is possible to speculate that the transposable element upstream of miR159c is at least partially responsible for the inability of the *MIR159c* locus to produce significant amounts of miR159c.

3.3.4 qSL-PCR provides an explanation for the phenotypes of *mir159* mutants

qSL-PCR is a relatively new method developed for miRNA quantitation that promises to offer several advantages over small RNA blots (Chen et al., 2005), in a conceptually similar way to the advantages of qRT-PCR over traditional northern blotting. In cases where only small amounts of RNA are available, many samples need to be assayed, or when absolute quantification is required, the qRT-PCR method may offer advantages. For this study, the qSL-PCR assays were useful beyond these obvious applications. Firstly they were able to show that miR159 in *mir159ab* has been reduced to negligible levels (<1.5% compared to wild-type), and thus provided a molecular explanation for the drastic phenotype seen in this mutant. Secondly, they were able to show that the endogenous level of mature miR159c is extremely low; reinforcing that miR159a and miR159b are by far the predominant members of this family. Thirdly they allowed for a rational interpretation of why the *mir159a-2* allele

produced no phenotype even when homozygous in the *mir159b* background, where quantitation of miR159a in this mutant showed a modest reduction (42% of wild type level) compared to *mir159a-1*.

The loss-of-function approach taken here has not only revealed the functional redundancy of miR159a and miR159b, but it has also been effective in highlighting the importance of miR159a and miR159b for *Arabidopsis* development. This contrasts with previously employed overexpression strategies, which have given no indication of the widespread nature of miR159 function. Indeed such studies have shown virtually opposite results, where anther/pollen defects appeared to be the only consequence of miR159 overexpression (Schwab et al., 2005). Now it can be shown that the functional importance of miR159a and miR159b extends beyond anther and pollen development. A further outcome of the *mir159ab* phenotype is it's similarity to transgenic plants that have had one of the miR159 target genes, *MYB33*, deregulated (Millar and Gubler, 2005; Palatnik et al., 2003). However there are many targets that miR159 could potentially regulate, and now the *mir159ab* mutant presents the opportunity to determine to what extent such targets may be regulated. This will be the focus of the following chapter.

Chapter 4

The functional specificity of miR159a and miR159b.

I'm fired up about the quad.

Tony Millar

4.1 Introduction

The *mir159ab* loss-of-function mutant provides a unique opportunity to determine the functional specificity of a miRNA(s). Previously, miRNA targets have been identified by several methods that will be discussed below. However compared to a genetic loss-of-function approach, where a miRNA can be removed from its natural context and the consequence of target deregulation determined, they all have limitations.

Upon discovery of plant miRNAs, one of the first features remarked upon that seemed noticeably different from animal miRNAs was their apparent high complementarity to potential mRNA targets (Reinhart et al., 2002). Combined with prior knowledge that siRNAs, which have perfect target complementarity, act through transcript cleavage, it was predicted and shortly after confirmed (Llave et al., 2002) that plant miRNAs can indeed guide cleavage of such high complementarity targets. Consequently one of the earliest benchmarks for target validation was the ability to recover cleaved transcripts that represented the 3' ends of target mRNAs sliced by RISC (Llave et al., 2002).

Recent deep sequencing technologies used in conjunction with 5'-RACE have allowed for a far more extensive interrogation of cleaved transcript populations (Addo-Quaye et al., 2008; German et al., 2008; Gregory et al., 2008). However analysis of transcript cleavage suffers from discriminating only one mode of miRNA operation, whereas there is now increasing evidence that translational repression is widespread in plants (Brodersen and Voinnet, 2009). Therefore regulation of a miRNA target exclusively by translational repression would not be detected using 5'-RACE methods. Furthermore 5'-RACE is a qualitative procedure that cannot determine the level of mRNA target cleavage by miRNAs.

A further approach taken to investigate miRNA:mRNA target relationships is miRNA overexpression (also see chapter 1). Although conceivably both protein and message levels could be measured in overexpression experiments, this has rarely be done in plants (Chen, 2004; Dugas and Bartel, 2008), and the notion that cleavage is the predominant mode of regulation has until very recently focused most overexpression studies on examining transcript levels. In any case this method of validation suffers from very obvious limitations, where constitutive overexpression is not reflective of the normal temporal/spatial expression patterns, or the *in vivo* levels of an endogenous miRNA. Therefore such approaches may over-represent the natural ability of a miRNA to down-regulate a particular target.

Finally, due to the apparently stringent sequence requirements for miRNA-mediated target cleavage, it has been relatively easy (at least for sequenced genomes such as *Arabidopsis*) to bioinformatically predict potential targets of a particular miRNA (Alves-Junior et al., 2009; Jones-Rhoades and Bartel, 2004; Rhoades et al., 2002; Wang et al., 2004; Zhang, 2005). However it is becoming clear that simple base pairing parameters are insufficient to predict *bona fide* miRNA:mRNA target interactions. For example in animal studies it has been shown that other determinants such as secondary structure around the target site, or specific binding proteins can influence the ability of a miRNA to down-regulate a particular target (Kedde et al., 2007; Kertesz et al., 2007). Accordingly increasingly complex models remain unable to perfectly describe the regulatory relationship between a particular miRNA and a potential target, as evidenced by the discovery of several non-predicted novel targets through deep sequencing augmented 5'-RACE (German et al., 2008).

Therefore in contrast to methods that contain assumptions not always reflective of an organism's biology, the loss-of-function approach represents an objective means of determining the functional specificity of a miRNA. By removing the miRNA from its cellular context, the level and consequence of a targets deregulation (if any) can describe the relative importance of miRNA regulation for a particular target. This is an important question to address, as it underlies the more central question still unanswered concerning the significance of miRNAs; what is the importance of miRNAs in gene regulation? By determining the functional specificity of miR159a and miR159b, this question may be answered for these miRNAs, and given that miR159 is widespread and highly conserved in the plant kingdom (Axtell and Bowman 2008), the answers may have resonance beyond *Arabidopsis*.

For miR159, bioinformatics initially predicted that the *GAMYB-like* genes can be regulated by miR159 (Reinhart et al., 2002) and the first 5'-RACE validated targets were *MYB33* and *MYB65* (Palatnik et al., 2003) (Table 4.1). A sequence alignment of all seven *Arabidopsis GAMYB-like* genes show they contain sequence conservation at the *MYB33*/*MYB65* miR159 binding site and therefore are all predicted to be targets of miR159. The importance of this site for miR159 regulation has been further demonstrated by mutating the miR159 target site in *MYB33* (*mMYB33*, a silent mutation) to render it resistant to miRNA regulation, resulting in pleiotropic developmental phenotypes in *mMYB33* transgenic plants (Millar and Gubler, 2005; Palatnik et al., 2003) (see also chapter 1).

Additionally miR159 was originally classified as part of a larger miR159/miR319 family (Jones-Rhoades and Bartel, 2004), where these miRNAs have highly similar mature miRNA sequences, although miR319 has been shown to predominantly regulate genes encoding *TCP* transcription factors (Palatnik et al., 2007). Indeed subsequent to this study, it has been shown by 5'-RACE cleavage assays that miR319 can occasionally target and cleave *MYB33/MYB65* (Palatnik et al., 2007). However the converse does not apply, as sequence differences prevent regulation of *TCP* transcripts by miR159 (Palatnik et al., 2007).

Further genes that do not belong to the *GAMYB-like* family have also been shown to be down-regulated by miR159 when it is over-expressed (Table 4.1). These were *ACS8* (At4g37770) and *OPT1* (At5g55930) (Schwab et al., 2005). Addtionally, the target *DUO1* (*MYB125*) has shown to be miR159 regulated based on the recovery of miR159 guided cleavage products (Palatnik et al 2007). Finally, the results of deep sequencing mediated analysis of 5'-RACE transcripts as described above have revealed an additional target, a copper/zinc superoxide dismutase (At5g18100) that is able to be cleaved by miR159 (German et al., 2008).

Therefore in *Arabidopsis* a range of targets have either been shown, or have the potential to be regulated by miR159 (Table 4.1). By using the *mir159ab* mutant the specificity and importance of miR159 in their regulation can be ascertained. This is the focus of this chapter: to determine to what extent these potential mRNA targets are regulated by miR159a/miR159b.

Name	Atg Ident	Sequence vs miR159: (a) AUCUCGAGGGAAGUUAGGUUU (b) UUCUCGAGGGAAGUUAGGUUU (c) UCCUCGAGGGAAGUUAGGUUU	Prediction/Verification
MYB33	At5g06100	UGGAGCUCCCUUCAUUCCAAU	ps/miRU/RH/R[1,2,3]
MYB65	At3g11440	UGGAGCUCCCUUCAUUCCAAU	ps/miRU/RH/R[1,2]
MYB101	At2g32460	UAGAGCUUCCUUCAAACCAAA	ps/miRU/RH/R[4,6]/O.E[3]
MYB104	At2g26950	UGGAGCUCCCUUCAUUCCAAG	ps/miRU/RH
MYB97	At4g26930	AUGAGCUCUCUUCAAACCAAA	ps/miRU/RH
MYB120	At5g55020	AGCAGCUCCCUUCAAGCCAAA	ps/miRU/RH/O.E[3]
MYB81	At2g26960	UCGAGUUCCCUUCAUUCCAAU	miRU/RH
DUO1	At3g60460	UGGAGCUCCAUUCGAUCCAAA	ps/miRU/R[5]/O.E[3]
TCP2	At4g18390	AGGGGGACCCUUCAGUCCAAU	miRU(159c)
ACS8	At4g37770	UCGAGUUUCUUUCAAUCCAAA	ps/miRU/R[6]/O.E[3]
OPT1	At5g55930	UAGAGCUUUCUUCAUUCCAAC	miRU/RH/O.E[3]
Zinc/Cu SODM	At5g18100	UGGACCUCACUUCAAUCCAUU	R[5]
MRG1	At2g34010	UAGAGCCCCCUUCAAACCAAA	ps/RH/R[5,6]
MRG-similar	At1g29010	UAGAGCCUCCUUCAAGCCAAA	ps/miRU
GAG-RetTrspn	At3g43684	AAUGGCUCCCAUCGAUCCAAA	ps/miRU
PHD(ATX3)	At3g61740	UAGAGCUCUCUUAAGUCUAAA	ps/miRU
Unknown Prot	At2g46890	CAGAGCUCUCUUCUAUUCAAU	ps/miRU
Anion/ExProt	At3g06450	AAGAGCUCCGUUCAGUCCACG	miRU
NPH3 Prot	At5g17580	AAAAGCUUCCUACGAUCCAAG	miRU(159b)
NAS2	At5g56080	UAGAGCUUUCUUGUAUCCAAU	miRU
Pseudogene	At3g46384	AAUGGCUCCCAUCGAUCCAAA	miRU
SPL	At4g27330	U AUGAGCUCUCUUCAAUCCAAA	RNAhybM
PPDK	At4g15530	G AAGAGUUUCCUUCAAUCCAAA	psRNA

Table 4.1: miR159 targets predicted by bioinformatics, and/or confirmed by RACE or over-expression.

All potential or verified targets of miR159a-c, from three different plant miRNA prediction bioinformatics programs (psRNA Target, miRU, modified RNA hybrid), and all published RACE and/or over-expression studies are shown. All mature miR159 members are shown 3'->5'. Target mismatches with miR159a are bold. Target bulges are shown in bold above the nucleotide 3' of the bulge. Bioinformatically identified targets specific for miR159b or miR159c are indicated with brackets in the last column.

Key: psRNA http://bioinfo3.noble.org/psRNATarget/ (Zhang 2005)
miRU http://bioinfo3.noble.org/miRNA/miRU.htm (Dai and Zao 2009)
RH http://bibiserv.techfak.uni-bielefeld.de/cgi-bin/rnahybrid_tdb_mirnas.cgi
(Alvez-Junior et al 2009). R= RACE, O.E = Overexpression.
1: Palatnik et al2003, 2: Palatnik et al 2007 3,Schwab et al 2005, 4 Reyes and Chua 2006, 5: German et al 2008, 6: Alvez-Junior et al 2009

The main aims of this chapter are:

(1) Determine what miR159 targets are transcriptionally deregulated in *mir159ab*

(2) Determine which miR159 targets are functionally important.

4.2 Results

4.2.1 *MYB33* and *MYB65* are deregulated throughout *mir159ab*

In *Arabidopsis* *MYB33* belongs to the seven member *GAMYB-like* family of transcription factors that all contain the conserved miR159 binding site (Table 4.1). This family also contains a conserved R2R3 DNA binding domain, and *MYB33* and *MYB65* have been shown to act redundantly (Millar and Gubler, 2005). Therefore it is possible that all seven *GAMYB-like* genes can not only function similarly, but are likewise regulated by miR159. Accordingly the expression of all seven *GAMYB-like* genes was examined for evidence of deregulation in the *mir159ab* mutant. RNA was extracted from a range of developmental stages in wild-type and *mir159ab Arabidopsis*: three-day-old seedlings, 19-day-old rosettes, inflorescences and siliques. In all these *mir159ab* tissues the uniform trend observed was 3- to 10-fold increases in steady-state mRNA levels of the redundant gene pair *MYB33* and *MYB65* (Fig 4.1), suggestive of their deregulation.

4.2.2 miR159a and miR159b act redundantly in controlling *MYB33* and *MYB65*

MYB33 and *MYB65* steady-state mRNA levels were also assayed in three-day-old seedling RNA isolated from the single *mir159a* and *mir159b* mutants. Transcript levels of *MYB33* and *MYB65* were mostly unaffected in these *mir159a* and *mir159b* backgrounds (Fig 4.1), indicating miR159a and miR159b are acting redundantly in controlling the levels of these transcripts. There was a minor increase in steady-state transcript levels of *MYB33*, *MYB65* and *MYB101* in *mir159a* single mutants, however these changes were not statistically significant ($P<0.05$, Students T-test). The lack of deregulation seen for these genes is reflective of the wild-type appearance of these single *mir159* mutants (chapter 3).

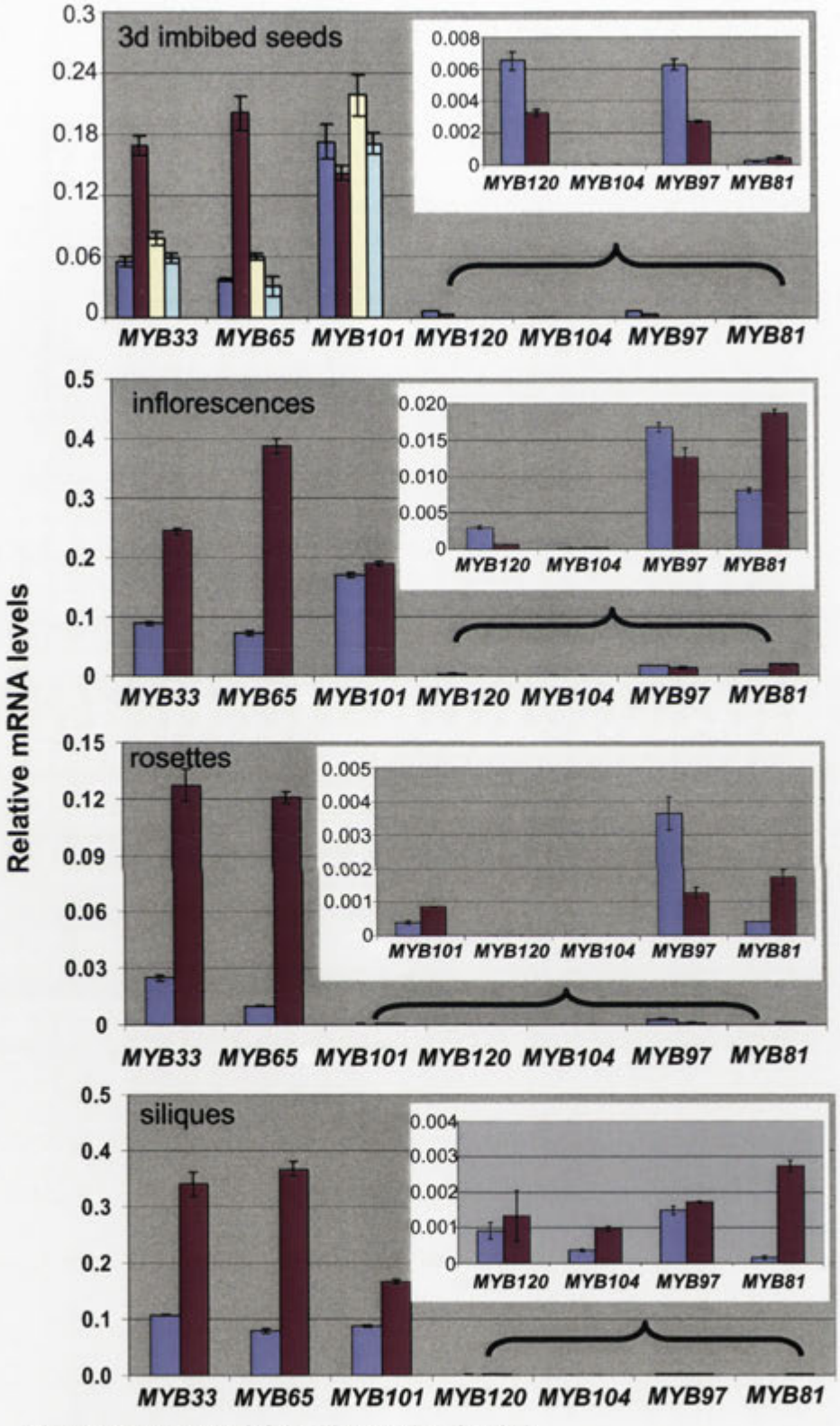


Fig 4.1: RNA levels of *GAMYB* genes in *mir159ab*.
qRT-PCR measurement was carried out on RNA extracted from different developmental stages. Wild-type is represented by blue bars and *miR159ab* by purple bars. *mir159a* is indicated by yellow bars and *mir159b* by light blue bars for analysis in 3-day imbibed seed. mRNA levels are shown relative to *Cyclophillin*. At least six biological replicates were pooled for each RNA sample. Error bars represent SEM.

4.2.3 Only *MYB33* and *MYB65* show consistently significant deregulation in *mir159ab*.

By contrast to *MYB33* and *MYB65*, there was little or no difference in *MYB101* expression levels between wild-type and *mir159ab* plants (Fig 4.1). In siliques and rosettes, *MYB101* levels were only two-fold higher. In rosettes, *MYB101* transcript levels were approximately 100-fold lower than *MYB33* and *MYB65*; therefore this expression level may not be of physiological significance. In inflorescences and three-day-old seedlings *MYB101* levels were unchanged.

The transcript levels of the other four *GAMYB-like* family members (*MYB81*, *MYB97*, *MYB104* and *MYB120*) are several orders of magnitude lower than *MYB33* and *MYB65*. Of the four genes, only *MYB81* has consistently higher transcript levels in *mir159ab* plants, however the *MYB81* increase in three-day imbibed seed was not statistically significant ($P < 0.05$ students T-test). The transcript levels of *MYB97* and *MYB120* were in fact lower in *mir159ab*. However, these relatively minor transcript level differences may reflect secondary effects due to the different morphologies of *mir159ab* and wild-type plants rather than miR159 regulation.

Several other non-*GAMYB-like* genes that contain potential miR159 target sites were assayed for deregulation in *mir159ab* (Fig 4.2). Similar to *MYB101* and the other anther/pollen predominant *GAMYB-like* genes, *TCP2*, *TCP4* and *DUO1* showed little evidence of the marked deregulation observed for *MYB33* and *MYB65*. There was a minor increase in *DUO1* transcript levels in the *mir159ab* inflorescences.

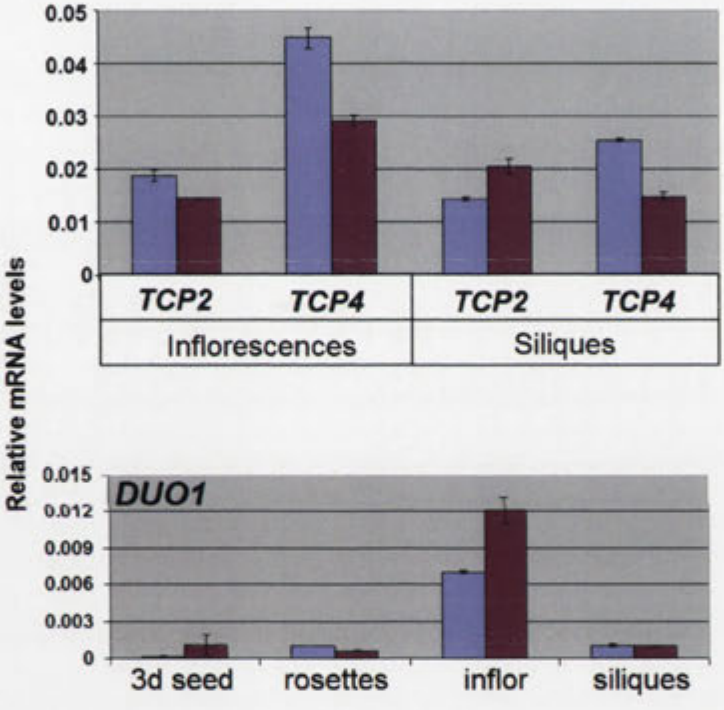


Fig 4.2: RNA levels of potential miR159 targets.
qRT-PCR was carried out on identical RNA as used for the *GAMYB-like* measurements. Wild-type is indicated by blue bars and *mir159ab* by purple bars. Error bars represent SEM. mRNA levels are shown relative to *Cyclophillin*.

4.2.4 The *MYB33:GUS* transgene is deregulated in *mir159ab*.

A *MYB33*:GUS transgene, (an in-frame translational fusion of MYB33 and GUS proteins, hence GUS activity is miR159 sensitive (Millar and Gubler 2005, see also chapter 1, Fig 1.2), was transformed into *mir159ab*. Ten *MYB33*:GUS (*mir159ab*) lines were generated and compared with four *MYB33*:GUS (wild-type) lines previously generated (Millar and Gubler 2005). In *MYB33*:GUS (wild-type), no GUS expression was observed in any developmental stage or tissue besides anthers, as also reported previously (Millar and Gubler 2005).

However *MYB33:*GUS in *mir159ab* showed strong GUS expression in all stages examined, which included five-day-old seedlings (Fig 4.3 A,B), ten-day-old rosettes (Fig 4.3 C), flowers (Fig 4.3 D) and siliques (not shown). This widespread expression of the MYB33:GUS protein in *mir159ab* is consistent with the broad deregulation of *MYB33* seen at the transcriptional level. Notably in wild-type, *MYB33* steady-state mRNA transcript was readily detectable in all developmental stages assayed (Fig 4.1) whereas *MYB33*:GUS protein is undetectable in wild-type except anthers (Millar and Gubler 2005, Fig 4.3 A). This result suggests that in addition to miR159-guided cleavage of *MYB33*, miR159 regulation of this gene may also occur though mechanisms that represses translation of any remaining transcripts.

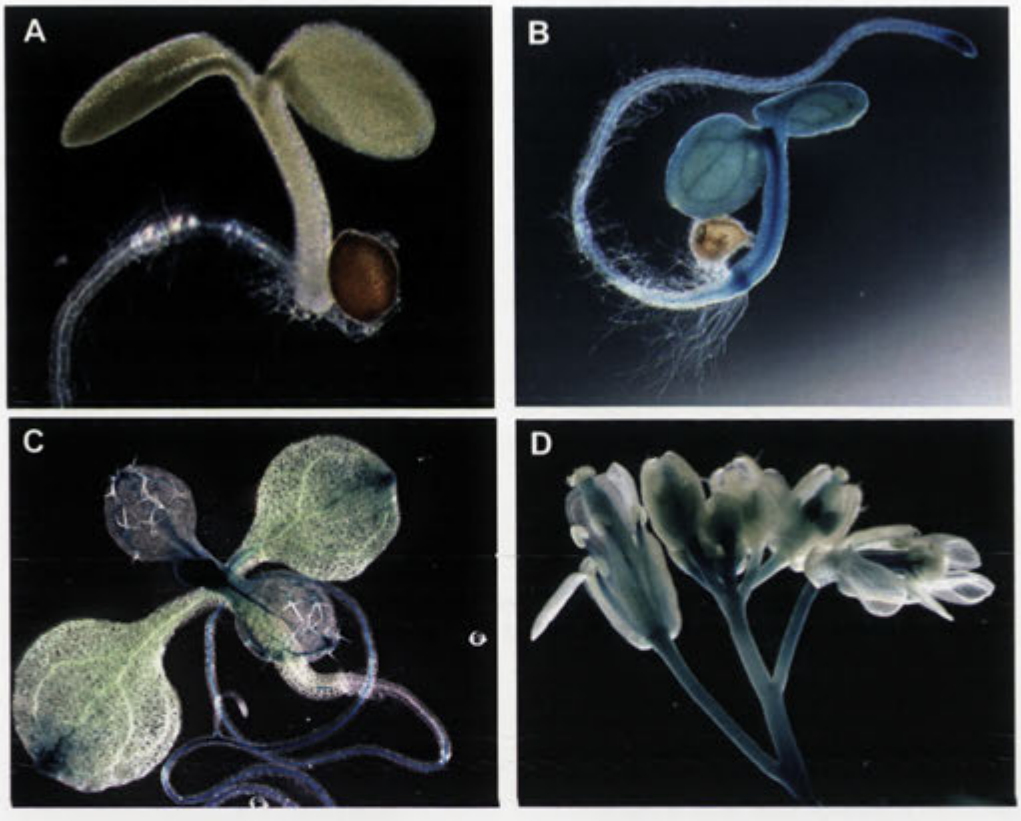


Fig 4.3 MYB33:GUS expression in wild-type and *mir159ab*. Overnight GUS staining was carried out on multiple lines (n=10 in *mir159ab*, n=4 in wild-type). (A) wild-type five-day-old seedling, (B) *mir159ab* five-day-old seedling. (C) *mir159ab* ten-day-old seedling. (D) *mir159ab* inflorescence.

4.2.5 Clustering analysis suggests two general classes of miR159 targets

The finding that only *MYB33* and *MYB65* appeared to be significantly deregulated consistently throughout *mir159ab* suggested other non-deregulated targets may not be co-transcribed with miR159a and miR159b. To investigate this further, clustering analysis was carried out using all the *GAMYB-like* targets and other targets that have been either confirmed by overexpression and/or 5'-RACE. Some of these targets are not available on the Affymetrix array and could not be included in the analysis. Nevertheless an interesting observation is that of the *GAMYB-like* genes, only *MYB33* and *MYB65* appeared to have widespread expression domains, whereas most of the other *GAMYB-like* targets appear to be expressed mainly in anthers and pollen, tissues where miR159a and miR159b appear to be absent (Fig 4.4).

Interestingly *MYB81* shows low expression in mature pollen and anthers, but may have a role in pollen development as indicated by its relatively high expression in sperm cells; this was supported by an additional online gene expression data-set that included transcript profiles for developing sperm cells (data not shown, http://bar.utoronto.ca/, (Winter et al., 2007)). These findings may explain why the *GAMYB-like* targets (*MYB81*, *MYB97*, *MYB101*, *MYB104*, *MYB120*) are not deregulated in *mir159ab* because they are transcribed in cells where miR159a/b are absent.

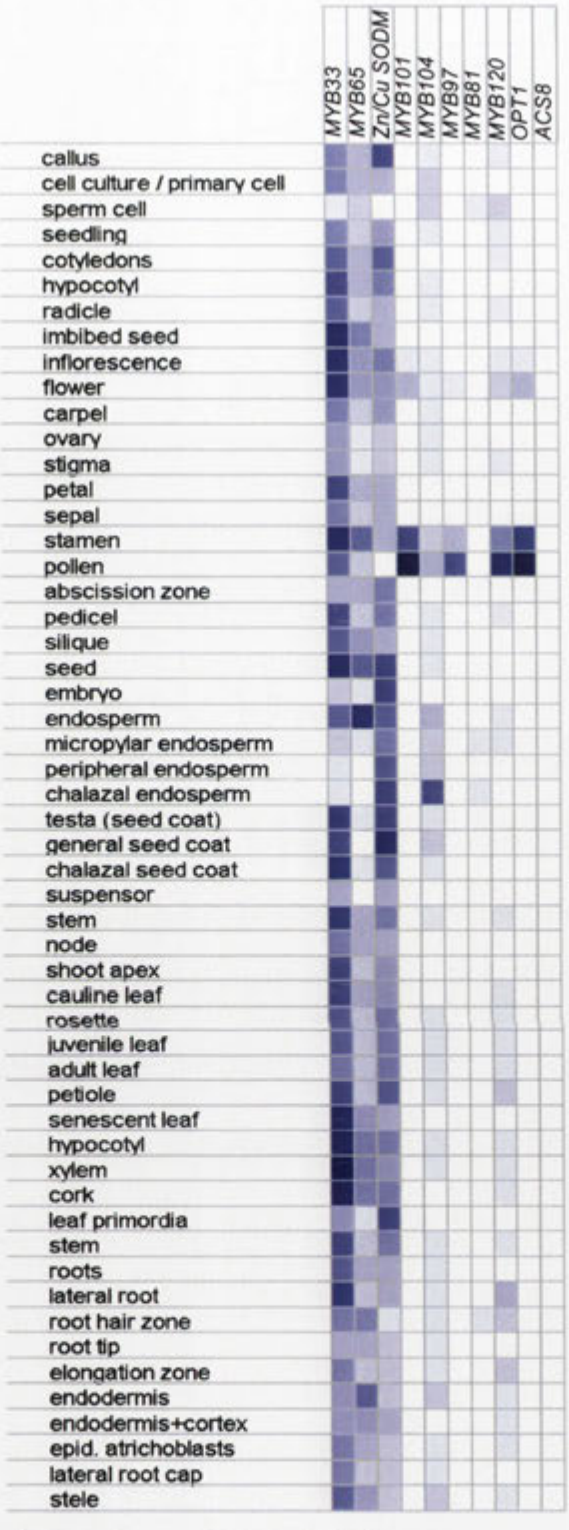


Fig 4.4 Affymetrix clustering analysis of miR159 targets.
Target genes were chosen based on satisfying at least one of three criteria: (1) belonging to the *GAMYB-like* family, (2) demonstrated downregulation by miR159 overexpression, (3) demonstrated cleavage by miR159 (see also Table 4.1). Not all targets were available on the array for clustering analysis. The heat map shows relative expression levels for all mRNAs that were available on the array (https://www.genevestigator.com/gv/index.jsp.)

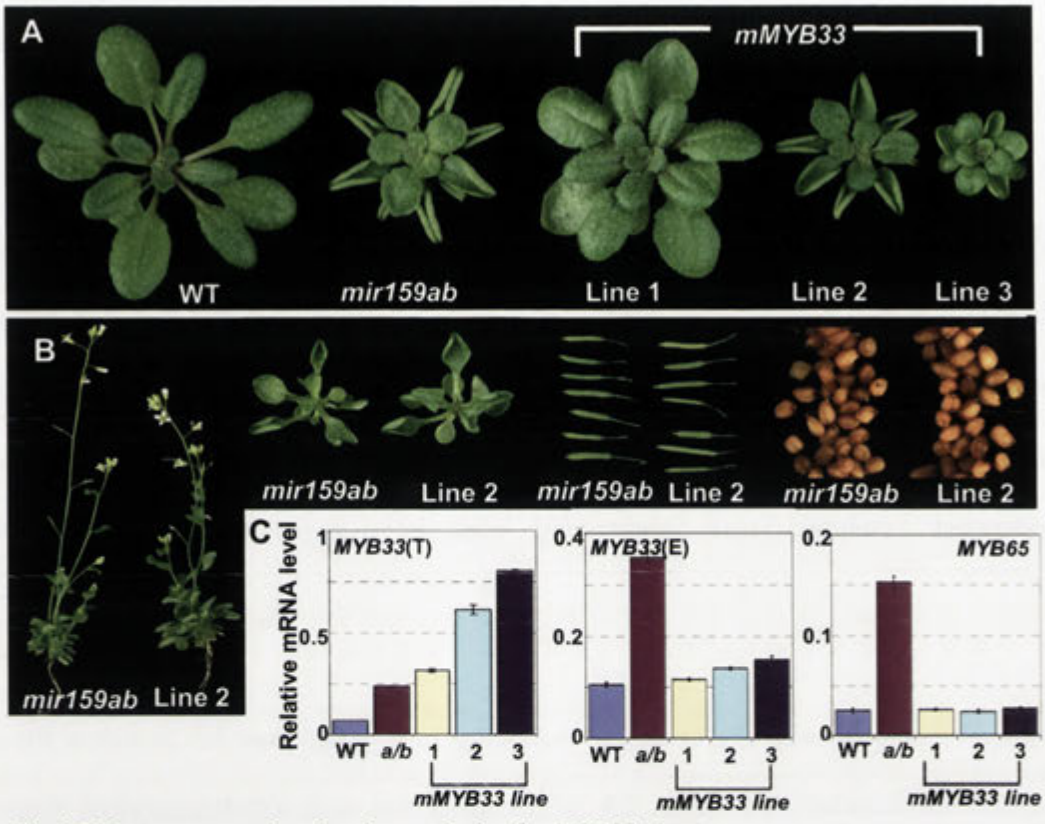


Fig 4.5 Phenotypes and molecular analysis of *mMYB33* plants

(A) Aerial views of rosettes of five week-old plants of wild-type, *mir159ab,* and three *mMYB33* lines grown under short-days. (B) Aerial views of rosettes of *mir159ab* and *mMYB33* (line2) grown under long-days. Also shown are siliques, seeds, and mature plants from the same lines. (C) qRT-PCR of cDNA from six-week-old plants was used to determine the relative expression of total (T) and endogenous (E) *MYB33* (using a primer to the miR159 target site that solely amplifies the wild type *MYB33* allele) and *MYB65* in wild-type (WT), *mir159ab* (*a/b*) and *mMYB33* lines 1-3. mRNA levels are shown relative to *Cyclophillin*. At least six biological replicates were pooled for each RNA sample. Error bars represent SEM.

4.2.6 *mMYB33* plants are phenotypically indistinguishable from *mir159ab*

The fact that the redundant gene pair of *MYB33/MYB65* showed consistent deregulation in *mir159ab* (Fig 4.1 and 4.3), suggested a specific regulatory relationship between these gene pairs. Furthermore miR159 resistant *mMYB33* transgenic plants (Millar and Gubler 2005) showed growth characteristics similar to that of *mir159ab,* suggesting that the deregulation observed in *mir159ab* is predominantly due to deregulation of *MYB33* and *MYB65*. Therefore for direct comparison, *mir159ab* was grown along side three independent *mMYB33* lines that displayed a weak (line 1), intermediate (line 2) and strong (line 3) phenotype, and their phenotypes were compared throughout development.

In all instances the morphologies of *mir159ab* and *mMYB33* (line 2) plants appeared largely indistinguishable from one another (Fig. 4.5 A, B). This includes the size and shape of the rosettes of short-day (Fig. 4.5 A) or long-day (Fig 4.4 B) grown plants. At bolting, the size and shape of inflorescences and siliques appeared indistinguishable (Fig 4.5 B) as did the seeds they set (Fig 4.5 B). The transgenic *MYB33* expression levels in these *mMYB33* lines were positively correlated with the severity of the phenotype (Fig. 4.5 C). However *mir159ab* did not conform to this correlation, reflecting that in addition to *MYB33*, the level of the redundant gene *MYB65* is also higher in *mir159ab*, but remains unchanged in the *mMYB33* lines (Fig 4.5 C). Therefore it is possible that total MYB33/MYB65 activity is at similar levels in *mir159ab* and *mMYB33* (line 2) plants.

4.2.7 *myb33* and *myb65* alleles suppress the *mir159ab* phenotype

The preceding data suggested that *MYB33* and *MYB65* deregulation is predominantly responsible for the *mir159ab* phenotype. To confirm this, *myb33* and *myb65* alleles were crossed into the *mir159ab* background. The probability of a homozygous F2 quadruple *mir159a/mir159b/myb33/myb65* mutant is 1/1024, which would be laborious to identify using PCR. However the absence of *MIR159a/MIR159b* leads to distinct upward curling leaves, therefore any F2 progeny of a *myb33/myb65* and *mir159a/mir159b* cross that showed leaf curling were expected to be homozygous for loss of *MIR159a* and *MIR159b*. Identification of such plants would facilitate identification of a quadruple mutant in the F3 progeny of *mir159ab* homozygous F2 parents.

Therefore the seed of a confirmed heterozygous *mir159a/mir159b/myb33/myb65* F1 plant was germinated and F2 plants which showed evidence of upward leaf curling were screened by PCR (Fig 4.6 A). Screening of sixteen F2 plants that showed various degrees of leaf curling revealed four plants that showed very subtle leaf curling had at least one wild-type copy of *MIR159a* or *MIR159b* (Fig 4.6 B). Examination of wild-type plants grown along side revealed that subtle leaf curling identical to these F2 progeny classified as "very mild" can occur occasionally in wild-type plants. However, all plants that showed more noticeable leaf curling, classified as either "mild" of "severe", were found to have no *MIR159a* or *MIR159b* alleles (Fig 4.6 C). Two of these eight F2 plants were identified that additionally contained at least one copy of *myb33* and *myb65*, and F3 plants were grown from their seed.

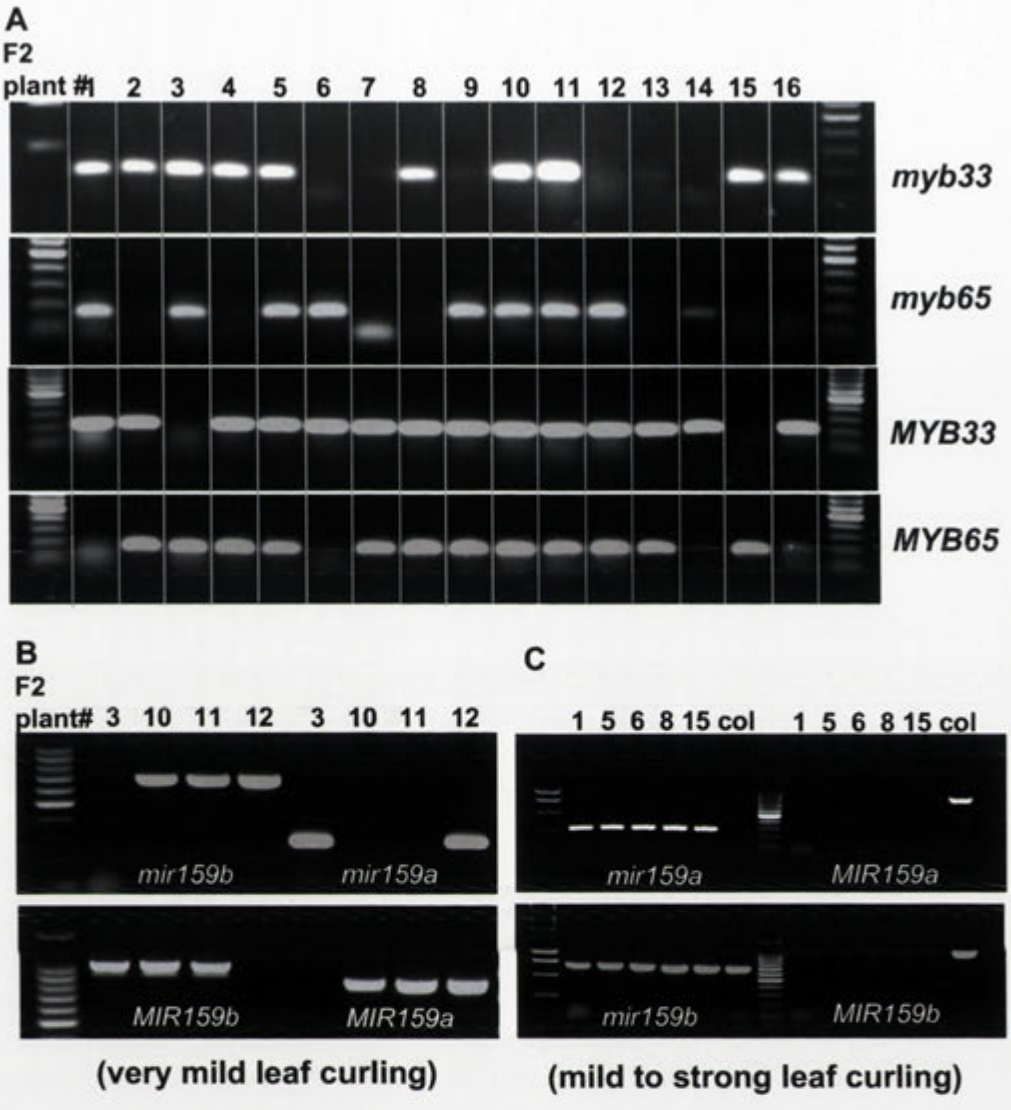


Fig 4.6 PCR genotyping of segregating F2 plants from the *mir159ab* x *myb33/myb65* cross. PCR was used to screen for the presence of *MYB33*, *myb33*, *MYB65* and *myb65* alleles in sixteen F2 segregants selected for signs of upward leaf curling (A). Plants were also concurrently screened for *MIR159a*, *mir159a*, *MIR159b* and *mir159b*. Plants 3, 10, 11 and 12 (B) that had marginally noticeable leaf curling (also seen occasionally in wild-type) all contained wild-type copies of *MIR159a* and/or *MIR159b*. Plants 1,5,6,8 and 15 all were homozygous for *mir159ab* (C) but had different combinations of *myb33/myb65* alleles; only plants 1 and 5 contained both *myb33* and *myb65* alleles (A) and were therefore chosen for subsequent F3 selection of the *mir159ab/myb33/myb65* mutant. There was contamination of the PCR for *mir159b* in wild-type (C), however both *MIR159a* and *MIR159b* alleles were clearly absent in plants 1,5,6,8,15 compared to their obvious presence in wild-type.

In this F3 population, a confirmed *mir159ab/myb33* triple mutant displayed a milder phenotype than that of *mir159ab* (Fig. 4.7), where growth was less stunted and leaf curling less severe. When a single *myb65* allele was present in the *mir159ab/myb33* mutant, leaving only a single wild-type allele of *MYB65*, leaf curling was even further reduced and far more subtle than in *mir159ab*. Finally, in a *mir159ab/myb33/myb65* quadruple mutant, all phenotypic characteristics of *mir159ab* were suppressed and the mutant appeared identical to *myb33/myb65*.

This reversion of the *mir159ab* traits in *mir159ab/myb33/myb65* demonstrates that *MYB33* and *MYB65* are solely responsible for the phenotype exhibited by *mir159ab* plants. Finally as the phenotype of *mir159ab/myb33* reflects only deregulated *MYB65* activity, this triple mutant confirms that *MYB65* regulates similar processes to that of *MYB33*. However in the *mir159ab* background *MYB33* and *MYB65* are no longer redundant, where their activity has become additive.

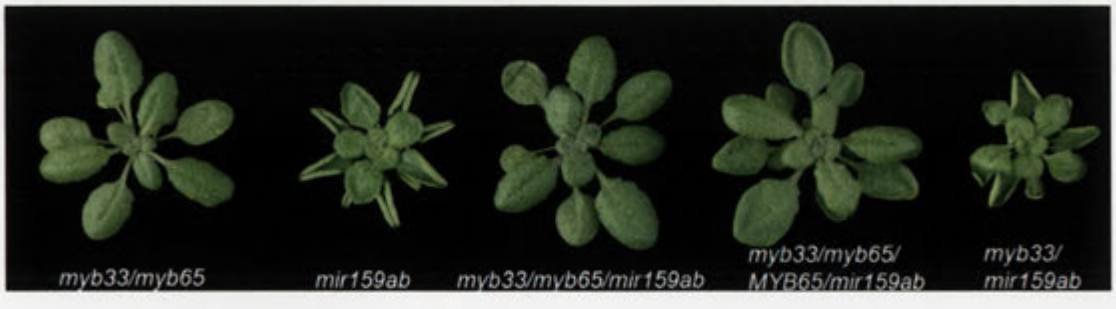


Fig 4.7 Suppression of the *mir159ab* phenotype
(A) Aerial view of rosettes of *myb33/myb65*, *mir159ab*, *myb33/myb65/mir159ab*, *myb33/myb65/MYB65/mir159ab* and *myb33/mir159ab* grown under short days for five weeks.

4.2.8 *MYB33* does not up-regulate *MIR159a* or *MIR159b*

It has previously been proposed that *MYB33* might be involved in positive feed-back regulation of miR159a and miR159b (Achard et al., 2004; Baulcombe, 2004). To investigate this possibility, the transcript levels of *MIR159a* and *MIR159b* were measured by qRT-PCR in the *mMYB33* lines used for analysis of transgenic and endogenous *MYB33* and *MYB65* levels (4.5). However there was no increase in *MIR159a* or *MIR159b* transcript levels in any of the *mMYB33* lines when compared to wild-type plants (Fig 4.8). In addition, there was no evidence of *MIR159* downregulation in the absence of *MYB33* and *MYB65*. On the contrary, *MIR159a* and *MIR159b* transcripts were slightly higher in *myb33/myb65*. However only *MIR159b* transcript was significantly higher than wild-type ($P < 0.05$ Students T-test). Previously, using a primer that distinguishes between endogenous and transgenic *MYB33*, it was found that the steady-state levels of endogenous *MYB33* were not reduced in the *mMYB33* lines (Fig 4.5 C). The fact that both endogenous *MYB33* and *MYB65* levels did not decrease in these *mMYB33* lines again supports the finding that higher miR159 levels are not present in the *mMYB33* lines.

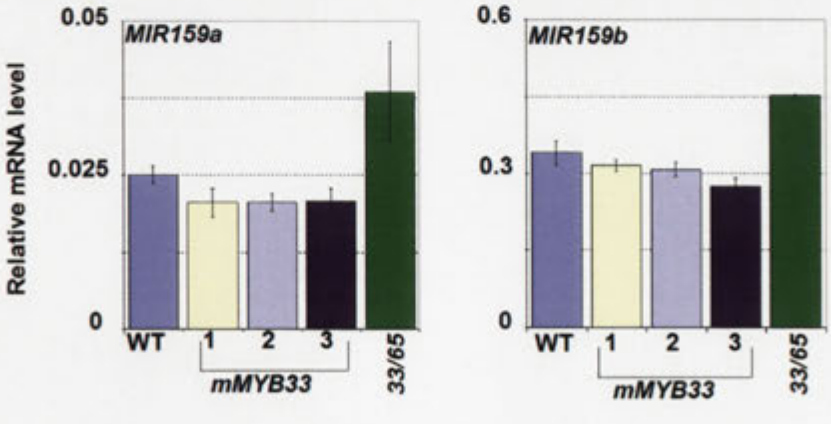


Fig 4.8 RNA levels of *MIR159a* and *MIR159b* in *mMYB33* lines
The RNA sampled from six week old long day grown *mMYB33* plants (lines 1,2,3 Fig 4.5) and the *myb33/myb65* mutant were assayed for *MIR159a* and *MIR159b* transcripts. mRNA levels are shown relative to *Cyclophillin*. Error bars represent SEM.

4.3 Discussion

4.3.2 miR159a and miR159b are functionally specific for *MYB33* and *MYB65*.

Bioinformatics approaches, overexpression strategies and isolation of miR159-guided cleavage products together predicted that the closely related *Arabidopsis MIR159* genes can regulate at least eight genes encoding MYB transcription factors, and further unrelated genes containing sequence motifs to which miR159 could potentially bind (Alves-Junior et al., 2009; German et al., 2008; Palatnik et al., 2003; Palatnik et al., 2007; Schwab et al., 2005). Through the genetic and molecular characterisation of *Arabidopsis* loss-of-function *mir159* mutants, it has been shown that the predominant role of miR159a and miR159b is to redundantly control just two of these genes, the redundant gene pair of *MYB33* and *MYB65*. This demonstrates a greater functional specificity than previously predicted.

4.3.3 Transcriptional domains determine miR159a/b specificity

There are several lines of evidence that can explain the functional specificity of miR159a/b. Firstly, from the previous chapter it was shown that *MIR159a:GUS* and *MIR159b:GUS* have widespread transcriptional domains (Fig 3.5). Similarly, *MYB33* and *MYB65* have widespread transcriptional domains (Fig 4.3 and 4.4). This would explain why *mir159ab* has global developmental defects. For instance in inflorescences, the only tissue where *MIR159a:GUS* and *MIR159b:GUS* was not expressed was in anthers, the sole tissue in which MYB33:GUS protein was expressed (Millar and Gubler 2005, Fig 1.2). In stark contrast, transgenic plants over-expressing a *35S:miR159a* transgene only produced anther defects (Schwab et al., 2005); constitutive miR159 expression would have little impact, as the transcriptional domains of

MYB33/MYB65 are already covered by endogenous miR159a/miR159b. This strong correlation between *MIR159a/b* transcription and action is in agreement with other miRNA systems that show miRNAs, unlike siRNAs, are unable to be transported from cell to cell, and their transcription matches their site of action (Alvarez et al., 2006; Parizotto et al., 2004; Tretter et al., 2008).

Consistent with these co-transcriptional domains, *MYB33* and *MYB65* transcript levels accumulate 3 to 10-fold higher throughout *mir159ab* plants. By contrast most other *GAMYB-like* genes do not appear to be deregulated, and this appears due to their transcriptional domains that predominantly reside in anthers/pollen, tissues in which miR159a and miR159b appear to be absent or very lowly expressed. This would explain why their expression levels do not dramatically increase in *mir159ab*. Of these *GAMYB-like* genes, only *MYB81* appeared to show evidence of consistent deregulation in *mir159ab*. However because *MYB81* is expressed at a level several orders of magnitude lower than *MYB33* and *MYB65*, these changes would be expected to make minor contributions the *mir159ab* phenotype, and this was confirmed by the wild-type appearance of *mir159ab/myb33/myb65* (see below). Additionally, it is possible the small changes seen for all these anther/pollen predominant *GAMYB-like* genes may represent the different morphologies of *mir159ab* and wild-type plants.

The apparent lack of transcriptional deregulation evidenced for the majority of these pollen/anther predominant *GAMYB-like* genes does not exclude the possibility that their regulation occurs through translational repression. However at least for miR159a /miR159b mediated regulation of these transcripts this would seem unlikely, because their transcriptional domains appear to be largely confined to anthers/pollen, where

miR159a and miR159b seem absent. Therefore the lack of mRNA deregulation seen for these *GAMYB-like* genes in *mir159ab* is likely due to non-overlap of transcriptional domains, and whatever regulatory mechanism miR159 might use has no direct consequence for their regulation.

4.3.4 *MIR159a* and *MIR159b* expression appears independent of *MYB33*

The strongly overlapping expression patterns of *mMYB33:GUS* (Millar and Gubler 2005) and *MIR159:GUS* (Fig 3.4) reporter genes suggested their transcription is controlled by a common regulator, and previously it had been shown that they can be induced by gibberellin (Achard et al., 2004). This led to the notion that *MYB33* expression could postively regulate *MIR159*. However the fact that steady state transcript levels of *MIR159a* and *MIR159b* were not elevated in *mMYB33* transgenic lines argues against this scenario. Supporting this, endogenous *MYB33* and *MYB65* steady state transcript levels were not lower in the *mMYB33* lines, indicating that mature miR159a/miR159b levels have remained unchanged.

4.3.5 Regulation of *MYB33* by miR159 occurs through two mechanisms.

The widespread GUS staining pattern observed for *MYB33:GUS* in *mir159ab,* compared with the total lack of MYB33:GUS activity in wild-type (except anthers) suggests that miR159a/b can act as a "switch" miRNA; acting to completely shut down expression of *MYB33:GUS*. This concurs with previous findings of Millar and Gubler 2005, where in wild-type, MYB33:GUS protein was undetectable in any tissue except anthers. By contrast, in all tissues examined in this study, *MYB33* transcripts were easily detectable, implying that in addition to cleavage, translational repression is used by miR159a/b to regulate *MYB33*. Comparison of *ago1* mutants deficient in slicing or

translational repression (Broderson et al., 2008) indicates the former mechanism is more critical for development. Although this study has only compared MYB33:GUS fusion protein with *MYB33* transcript, its suggests that translational repression of *MYB33* plays a significant role in the regulation of this gene. Accordingly it would be interesting to determine the relative contribution, and biological significance of slicing versus translational repression in the regulation of *MYB33/MYB65* by miR159a/b.

4.3.6 The functional specificity of miR159a/b is narrower than predicted.

The notion of a narrower miR159a/b functional specificity than has been previously predicted was strengthened by comparing the similarities of *mMYB33* transgenic plants with the *mir159ab* mutant. The fact that transgenic *MYB33* was higher in *mMYB33* plants identical to *mir159ab* further validated the notion that the *mir159ab* phenotype was due not only to deregulation of *MYB33*, but also its redundant gene partner *MYB65*. The previous assumption that *MYB65* transcription and function is similar to *MYB33* was further supported by the observation that both these genes had virtually identical patterns of deregulation in *mir159ab*. Furthermore, *mir159ab/myb33* plants that contained wild-type *MYB65* showed leaf curling similar but less severe to *mir159ab,* demonstrating that *MYB65* can function in the same manner as *MYB33*. Finally the *mir159ab/myb33/myb65* quadruple mutant established that despite the possibility that miR159 may regulate many potential genes, the only targets of *in vivo* relevance are *MYB33* and *MYB65*.

Previously using overexpression strategies and transcriptome analysis it was shown that plant miRNAs appear to only have a limited number of targets which they regulate. The loss-of-function strategy employed here shows miR159a/miR159b predominantly

regulate *MYB33* and *MYB65*, whereas most other predicted targets are mainly transcribed in tissues where the miRNAs are absent. There is only one 5'-RACE confirmed target- a Zn/Cu Superoxide dismutase, that appears to be an exception to this scenario (Table 4.1). However, at least under the standard conditions used in this study and in relation to development, miR159 regulation of this gene is apparently of no biological consequence, as evidenced by the wild-type appearance of the *mir159ab/myb33/myb65* quadruple mutant.

The wild-type phenotype of *mir159ab/myb33/myb65* (except in anthers, that retain the *myb33/myb65* phenotype) would argue that deregulation of low complementarity miR159 targets either does not occur or is sufficiently minimal to produce no observable biological consequence. Although translational repression of a low complementarity target in plants has been demonstrated for a single miRNA (Dugas and Bartel, 2008), this study reinforces more widespread reports that demonstrate regardless of their mode of operation, plant miRNAs require high target complementarity to regulate them (Alvarez et al., 2006; Palatnik et al., 2007; Schwab et al., 2006; Schwab et al., 2005).

4.3.7 Despite sequence differences miR159a and miR159b show no evidence of subfunctionalisation

The fact only homozygous *mir159ab* shows a mutant phenotype demonstrates that both *MIR159a* and *MIR159b* are able to carry out the same function (Chapter 3). Examination of miR159a and miR159b sequences compared to *MYB33/MYB65* shows

that miR159b contains an additional target mismatch compared to miR159a (table 4.1). However despite this difference, miR159b appears functionally equivalent to miR159a, given that at a predicted level of 15% in a *mir159a/MIR159b/mir159b* mutant, miR159b is able to downregulate *MYB33/MYB65* to the extent no mutant phenotype is apparent. This demonstrates the mismatch at this position is functionally inconsequential, and *MIR159a* and *MIR159b* have not subfunctionalised.

4.3.8 A discrete range on relevant *in-vivo* targets is an emergent theme in plant and animal miRNA function

The discrete specificity of miR159a/b is also similar to the few examples of miRNA loss-of-function mutants characterized to date. In plants it was suggested that for the *mir164abc* loss-of-function mutant, only two of the targets of miR164 were likely to account for the majority of the phenotypic changes in *mir164abc* plants. This was based on close phenotypical similarities of plants expressing miR164 resistant *CUC* targets and *mir164abc* mutants. (Sieber et al., 2007). In nematodes, although *lin-4* and *let-7* are predicted to regulate many genes, either mutant can be suppressed through the mutation of single target genes (Ambros, 1989). Likewise in *Drosophila*, removal of a single target of miR279 significantly corrects the miRNA loss-of-function phenotype (Cayirlioglu et al., 2008). Finally in mice, although hundreds of genes contain a conserved miR150 binding site, restoration of the *mir150* knockout phenotype could occur by deregulation of a single target alone (coincidently a *MYB* transcription factor) (Xiao et al., 2007). Thus a greater functional specificity for miRNA targets than predicted by bioinformatics or observed in overexpression experiments is an emergent and unifying trend in both plant and animal miRNA studies.

One explanation for these observations is that the miRNAs and their targets are transcribed in adjacent but mutually exclusive expression zones, where it is thought that the role of the miRNA is to provide genetic buffering to ensure accuracy to gene expression programs (Bartel, 2004). Similarly miR159a and miR159b may have a dual role, where they cleave transcripts of *MYB33* and *MYB65* in tissues in which they are co-transcribed, and secondly where it ensures that other targets with non-overlapping transcriptional domains are restricted to those tissues. This may explain the presence of miR159 target sites in the *GAMYB-like* genes that are apparently not targeted by miR159a or miR159b. Alternatively, the presence of these putative target sites may be required for regulation by miR159c. In any case, the presence and conservation a miR159 site in other genes that are not deregulated in *mir159ab* raises the question of its biological relevance. This question will be addressed in the following chapter.

Chapter 5

Investigating miR159 regulation

of anther and pollen transcribed target genes.

If you want to go to the trouble of doing anther sections you'd better have a bloody good question

-Rosemary White

5.1 Introduction

The presence of evolutionarily conserved miR159 target sites in the *Arabidopsis GAMYB-like* genes suggests their regulation by miR159 is important. Over a large time scale this seems valid; for the moss *Physcomtrella patens,* an ancient form of miR159/miR319 has been shown to cleave a *MYB* gene transcript (Axtell et al., 2007), and at a closer evolutionary distance, the miR159 site in rice *GAMYB* is inferred to be important because overexpression of miR159 downregulates *GAMYB,* causing anther defects (Tsuji et al., 2006)

Unlike in rice (Tsuji et al., 2006) *Arabidopsis* miR159a/b expression is absent or very low in anthers (Allen et al., 2007). This is supported by several lines of evidence. Firstly *mir159ab* pollen appears wild-type which contrasts to the otherwise pleiotropic developmental defects throughout this mutant, consistent with the widespread expression domain of miR159a/b (Chapter 3). Secondly, transgenic 35S:miR159a *Arabidopsis* plants are male sterile, indicating endogenous miR159a and miR159b expression domains limit their ability to affect anther and pollen development (Schwab et al., 2005). Yet the fact that all eight *MYB* genes (the seven *GAMYB-like* genes and *DUO1*) with conserved miR159 binding sites are transcribed in anthers/pollen (chapter 4, and see Fig 5.1) presents an obvious question: what is the relevance of miR159 target sites in genes that are predominantly expressed in anthers/pollen, that appear not to be regulated by the major miR159 members, miR159a and miR159b?

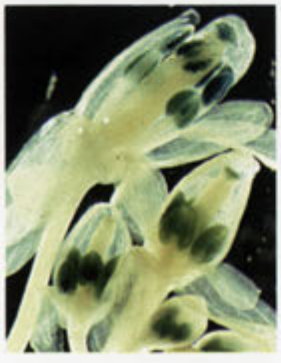


Fig 5.1 *MYB101* Promoter:*GUS* expression in anthers
The GUS expression pattern for *MYB101*pro:GUS shows expression in anthers, where *MIR159a* and *MIR159b* appear not to be expressed (see Fig 3.5) Image provided by Anthony Millar (Allen et al., 2007)

An obvious possibility is that these genes are regulated by miR159c, and this will be explored in the next chapter. It is also possible that the closely related miR319 family is responsible for regulation of these anther/pollen transcribed *MYB* genes. However, it appears that miR319 predominantly targets gene members of the *TCP* family (Palatnik et al., 2007), and this regulatory relationship is conserved in other species (Ori et al., 2007). Nevertheless it has been shown that miR319 can indeed regulate *MYB33* and *MYB65*, but this regulation appears minor compared to miR159, due to a much smaller expression domain and a lower expression level of miR319 compared to miR159 (Palatnik et al., 2007).

Another possibility is the miR159 target site ensures *GAMYB-like* expression is restricted to anthers and pollen. Thus any aberrant *GAMYB-like* transcription is down-regulated post-transcriptionally by miR159a/b and possibly miR159c, where at least miR159a and miR159b have been shown to be strongly expressed outside anthers. This scenario has recently been shown to occur in *Arabidopsis*, where miR395 is expressed in xylem adjacent to the target *SULTR2*, which is only transcribed in phloem companion cells (Kawashima et al., 2009). In this situation, where miRNAs and their mRNA targets are expressed in mutually exclusive domains, miRNAs act as a "backup" form of regulation, secondary to transcriptional control of an mRNA target.

Therefore there are numerous possibilities as to why anther/pollen transcribed *GAMYB-like* genes contain miR159 target sites. Accordingly this chapter has two related aims: (1) Determine if miR159/miR319 regulation of the *GAMYB-like* genes expressed predominantly in anthers and pollen occurs, and (2) if such regulation occurs, to determine its biological significance.

5.2 Results

5.2.1 miR159 regulation extends beyond *MYB33* and *MYB65*.

The 5'-RACE recovery of degraded mRNAs whose first ten nucleotides corresponds to complimentary miRNA binding sites has been previously used to demonstrate miRNA-guided cleavage and therefore regulation (Llave et al 2002) (Fig 5.2). Similarly, to determine if miR159 regulation extends beyond *MYB33* and *MYB65*, 5'-RACE analysis of *GAMYB-like* targets with conserved miR159 binding sites was carried out. Inflorescence tissues were chosen as they contain anthers/pollen, tissues in which all of these *GAMYB-like* genes are strongly expressed. Furthermore to gain insight into what extent miR159c-guided cleavage may be involved in regulating these *GAMYB-like* genes, 5'-RACE was carried out on both wild-type and the *mir159ab* mutant. Finally any miR319-guided cleavage could be determined by the 5'-RACE assays, as mature miR319 is only 20 nucleotides in length and thus will guide cleavage one nucleotide upstream of the miR159 cleavage position (Palatnik et al., 2007).

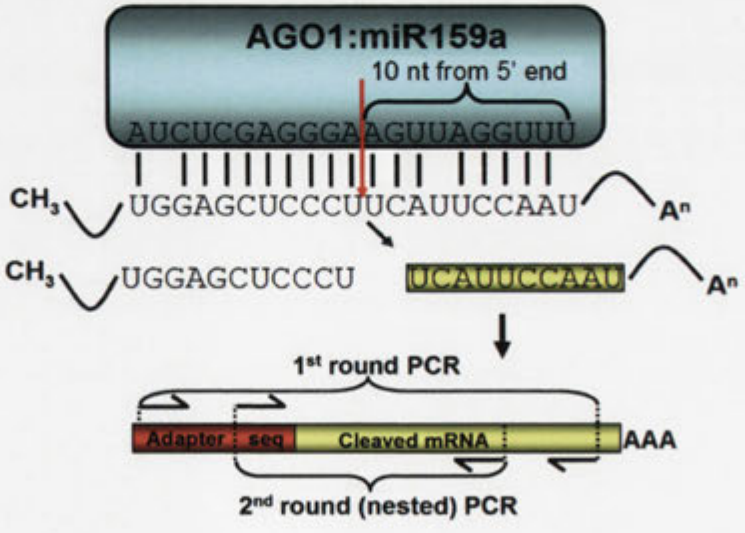


Fig 5.2 Schematic representation of modified 5' RACE.
miR159 guided cleavage products are generated by AGO1 mediated slicing of the phosphodiester bond 10 nt from the 5'end of the miRNA (here miR159a cleavage of *MYB33* is used as an example). A ribo-oligonucelotide adapter (red box) is ligated to the 5' cleaved RNA fragment (yellow box). Two rounds of PCR (nested PCR) are used to recover miR159 guided cleavage products.

Because *MYB33* has previously been validated by 5'-RACE as a target in wild-type, it was chosen in this study as a technical control, and to compare the efficacy of cleavage product recovery against the different *GAMYB-like* genes. The *MYB33* 5'-RACE reaction (Fig 5.3) produced fragments of the predicted size for miR159-guided cleavage of *MYB33* in both the first and second (nested) rounds of PCR, and as expected, most (8/9) clones corresponded to fragments indicating miR159-guided cleavage.

For *mir159ab*, the *MYB33* nested PCR band appeared slightly fainter than in wild-type (Fig 5.3). Only a single clone (1/7) was recovered that corresponded to miR159-guided cleavage, and another single clone was found corresponding to miR319-guided cleavage. The remaining five clones mapped elsewhere in the *MYB33* transcript, suggesting these have arisen through a non-miRNA mediated degradation mechanism. As only single clones corresponding to putative miR159- and miR319-guided cleavage were found, this raises the possibility they are in fact coincidental non-miRNA degradation products.

For *MYB101* in wild-type, the first round of 5'-RACE produced an extremely faint smear (Fig 5.3). Because only the reverse primer has unique specificity, this is not unusual for such reactions. The second (nested) PCR reaction resulted in a smear that had a very slight increase in concentration of staining near the size expected for the nested product, but this was quite diffuse, extending above and below the expected size range. After cloning and sequencing, 19 of the 29 cloned inserts corresponded to miR159-guided cleavage products, whilst the remaining clones mapped elsewhere.

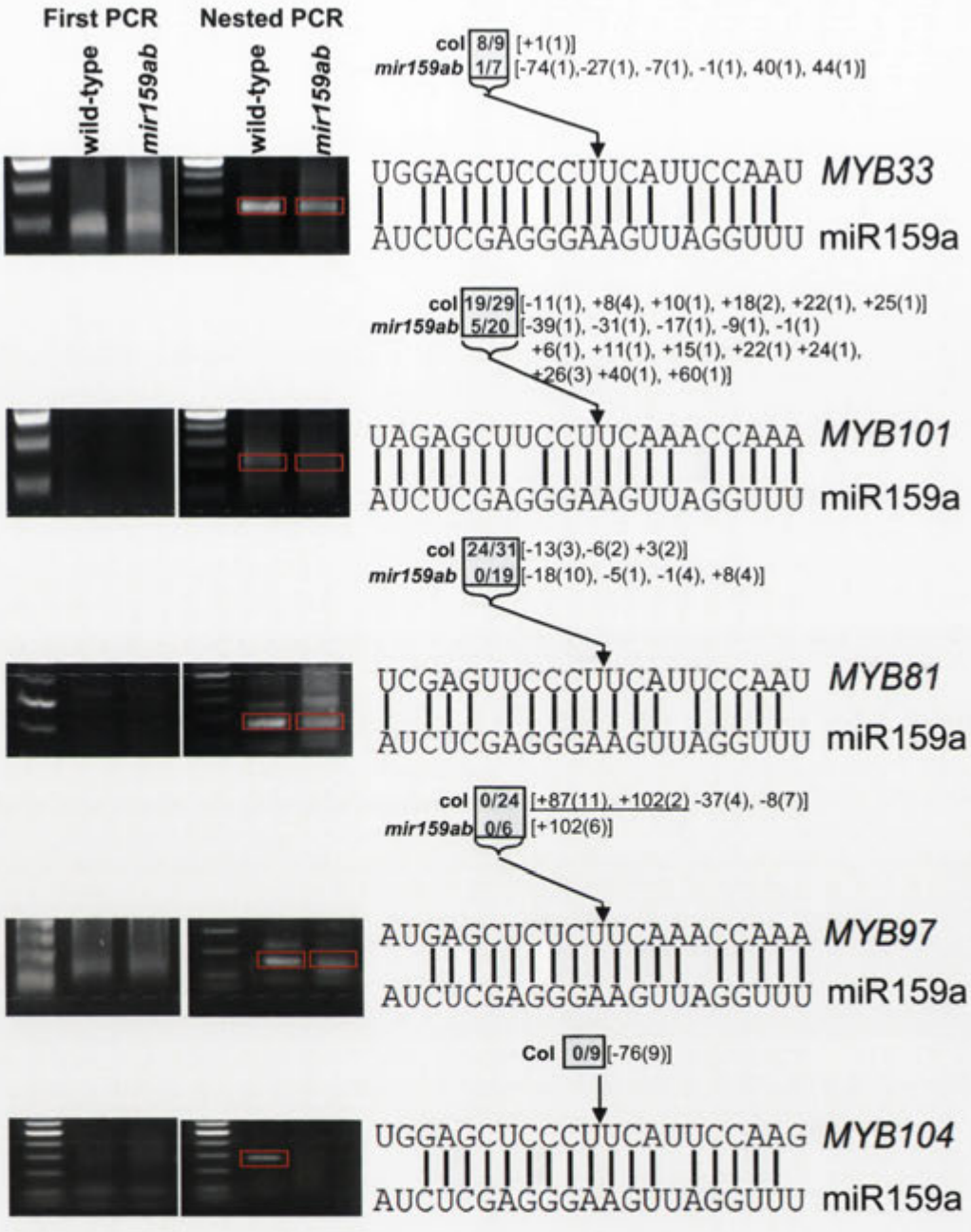


Fig 5.3 Modified 5'-RACE cleavage assays of *GAMYB-like* genes
Inflorescence purified mRNA from wild-type and *mir159ab* was subject to modified 5'-RACE PCR. The products from first round and second (nested) PCR were run on agarose gels. Red boxes indicate approximate regions that were gel purified. Clones of gel-purified products from the nested PCR reactions were sequenced. The sequence similarity of each *GAMYB-like* target is shown compared to miR159a. Numbers in boxes indicate the proportion of clones out of the total number analysed that mapped to the miR159 cleavage position (indicated by arrow). Numbers inside square brackets indicate the position of any further clones relative to the miR159 cleavage site, with (+) numbers indicating fragments that are further downstream of the miR159 cleavage site and (–) numbers indicating fragments that map upstream of the miR159 cleavage site. Numbers in round brackets represent the number of clones found at each position. For *MYB97*, a second RACE PCR was performed (not shown) and produced smaller fragments that are indicated by the underlined numbers.

The high proportion of recovered clones corresponding to miR159-guided cleavage site would argue that they represent genuine miR159-guided cleavage products, and are not coincidental degradation products of *MYB101*. However the fact that the 5'-RACE assay was clearly not as efficient as for *MYB33*, combined with the number of other transcripts not corresponding to miR159-guided cleavage fragments, would imply that cleavage products of *MYB101* are rarer than for *MYB33*.

In *mir159ab*, the *MYB101* 5'-RACE PCR product appeared less concentrated than in wild-type in the predicted size range (Fig 5.3). Sequencing revealed only 5 of 20 clones corresponded to miR159-guided cleavage fragments of *MYB101*, with the remaining clones mapping elsewhere (Fig 5.3). Therefore in comparison to wild-type, a smaller fraction of *MYB101* 5'-RACE products were representative of miR159-guided cleavage.

For *MYB81*, in both wild-type and *mir159ab* the first round of PCR produced PCR products that were significantly larger than expected and the second round produced a number of different sized products (Fig 5.3). For wild-type, the strongest PCR product was in the predicted size range, and for *mir159ab*, a similar sized product was present but was less abundant and other products were made of differing sizes but of similar abundance. Sequencing revealed 24 out of 31 clones corresponded to potential miR159-guided cleavage products in wild-type. Although no clones corresponded to potential miR159-guided cleavage products in *mir159ab*, four clones mapped to the miR319-guided cleavage site.

This result continues the trend seen for *MYB33* and *MYB101*, where miR159-guided cleavage products for all three *GAMYB-like* genes are more difficult to recover in *miR159ab* than in wild-type. This suggests that miR159a and miR159b are the major cleavage regulators of all three genes.

In stark contrast to *MYB33*, nested PCR was required to generate PCR products of the correct size range for *MYB101* and *MYB81*, and for the latter, multiple sized products were generated in the nested PCR reaction. This suggests the abundance of miR159-guided cleaved transcripts of *MYB81* and *MYB101* are substantially lower than that for *MYB33*.

For both *MYB97* and *MYB104*, no cleavage products were found in either wild-type or *mir159ab*, and neither 5'-RACE reaction produced discrete bands in the size range expected (Fig 5.3). For *MYB97*, several different 5'-RACE primer combinations were used but all were unsuccessful in finding any evidence of miR159-guided cleavage for this gene. For *MYB104*, only the wild-type sample produced a product, but clones from this all mapped 76 bp upstream of the predicted miR159-guided cleavage site.

In conclusion, because of the detection of miR159-guided cleavage products, *MYB101* and *MYB81* would be considered as miR159-regulated genes. Although the 5'-RACE data is not quantitative, the trends in the data suggest that miR159a and miR159b are the predominant cleavage regulators of these genes.

5.2.2 Cleaved *GAMYB-like* mRNAs are rare in comparison to uncleaved mRNAs.

Because the 5'-RACE experiments only qualitatively indicated miRNA-guided cleavage, qRT-PCR on *GAMYB-like* transcripts were undertaken in an attempt to quantitate the extent that miRNA-guided cleavage products comprise the total steady-state levels of each gene. Two sets of qRT-PCR primers were used; one that spanned the miR159 target site (uncleaved) and another downstream of the target site (total) that would detect all messages including any intact 3'-end cleaved mRNA (Fig 5.4).

For *MYB33*, qRT-PCR on cDNA prepared from three different tissues consistently showed that fold-changes from wild-type to *mir159ab* were similar, regardless of whether the uncleaved or total primer sets were used (Fig 5.4). If cleavage products were accumulating to significant levels, it would be expected that the ratio of *MYB33* transcripts in wild-type versus *mir159ab* using the total primer set would be lower that the ratio using the uncleaved primer set. This is because the total primers can measure both pools of mRNA (cleaved and uncleaved) and so should be significantly higher in wild-type. However this is not reflected in the qRT-PCR measurements as the ratios were nearly identical in all tissues.

To test if this trend occurred for the other *GAMYB-like* genes for which miR159-guided cleavage products were isolated; *MYB65*, *MYB81* and *MYB101* were all assayed using both total and uncleaved primer sets in tissues where these genes had shown increased levels of steady-state transcripts in *mir159ab* (chapter 4), and hence miR159-guided cleavage may be a major regulatory mechanism. In all instances, there were no major differences in the ratios of mRNA levels as determined by using the uncleaved and

total primer sets (Fig 5.4). Thus the trend seen for these *GAMYB-like* genes was consistent regardless of the gene assayed or tissue chosen.

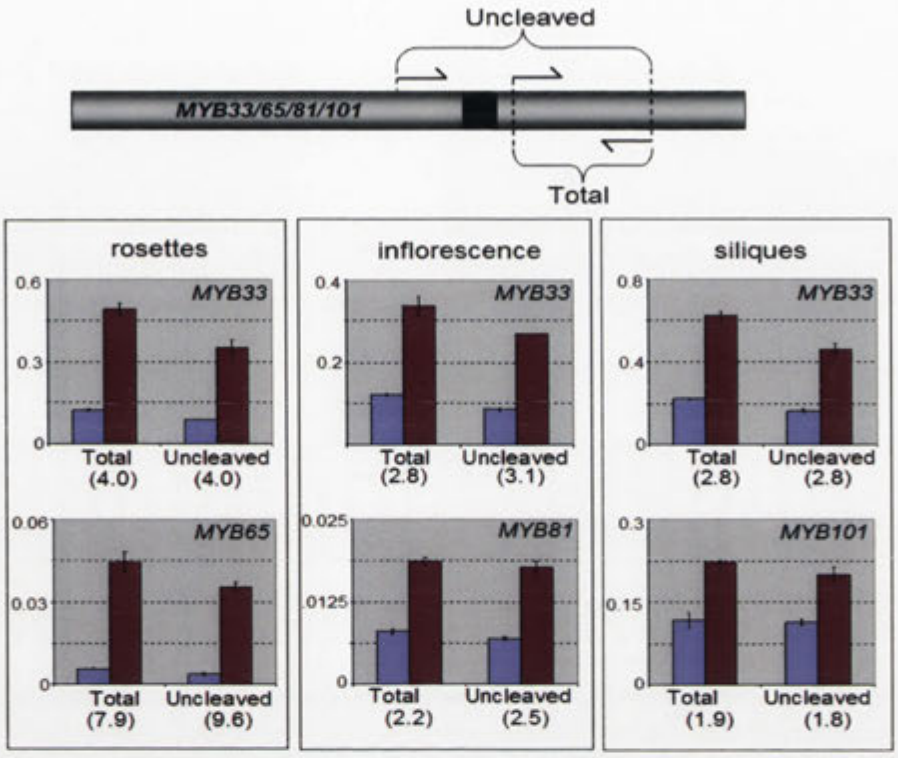


Fig 5.4 qRT-PCR analysis of un-cleaved and total transcripts.
RNA was extracted from various tissues and subject to qRT-PCR. The same samples were tested for gene expression with primers to total and cleaved transcripts for different *GAMYB-like* genes. The black box schematically represents the miR159 target site in relation to the primer sets. Blue bars=wild-type and purple bars= *miR159ab* mutant. Values represent mRNA levels relative to *Cyclophillin*. The ratios of *mir159ab* to wild-type mRNA expression levels are shown in brackets. At least six biological replicates were used for each RNA sample. Error bars represent SEM.

Therefore although the 5'-RACE assays were able to demonstrate that miR159-guided cleavage occurs for certain *GAMYB-like* genes, qRT-PCR measurements suggest that the resulting cleavage products are rare in comparison to total transcripts. The fact that nested PCR was required to visualize potential cleavage products with the 5'-RACE analysis, taken together with this qRT-PCR data, indicate that miR159-guided cleavage products of these *GAMYB-like* genes represent a very small fraction of their total steady-state transcript levels.

5.2.3 Analysis of plants over-expressing *MYB101* or miRNA-resistant *mMYB101*

The previous data suggests that the *GAMYB-like* genes, *MYB101* and *MYB81* are regulated by miR159 and possibly miR319. Although all the phenotypes of *mir159ab* are suppressed in the *mir159ab/myb33/myb65* mutant, similar to *myb33/myb65* this quadruple mutant is male sterile, and therefore miRNA-regulation of these additional *GAMYB-like* genes may be required for anther development. As *MYB101* is the highest expressed *GAMYB-like* gene in anthers and pollen (Fig 4.4, see also Fig 5.1), it was chosen to address this question.

Firstly, a genomic clone containing the entire *MYB101* gene was generated. This included 3232 bp of genomic sequence upstream of the start codon, extending to the next adjacent gene, and 1162 bp of sequence downstream of the stop codon to the next adjacent gene (Fig 5.5). The entire length of genomic sequence used was 6333 bp. As the complete flanking sequences were used (including the expected native promoter region), transcriptional regulation of the *MYB101* transgene would be expected to be similar to endogenous *MYB101*.

To generate the mutant *MYB101* transgene (*mMYB101*), site directed mutagenesis was used to make eight bp changes to completely disrupt miR159 regulation while conserving the wild-type protein coding sequence (Figure 5.5). The changes were made throughout the entire miRNA target site to avoid the possibility of sequestering miR159 by acting as a target "mimic" (Franco-Zorrilla et al., 2007). Comparison of anther development between transgenic *MYB101* and *mMYB101* plants should elucidate any critical miRNA regulation of this gene.

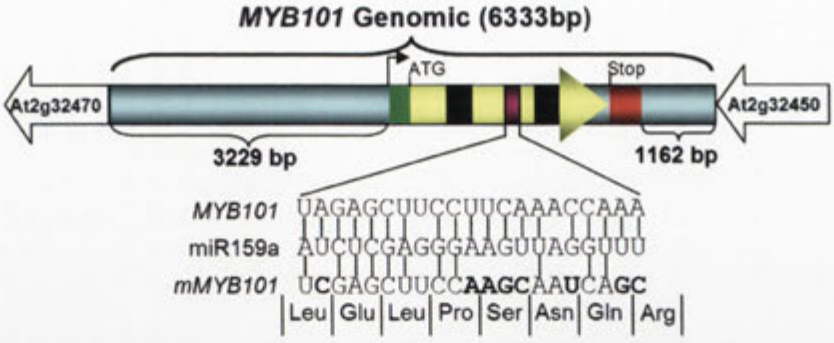


Fig 5.5 The genomic *MYB101* construct used to transform Arabidopsis.
6333 bp of genomic sequence was amplified from *Arabidopsis* and included the entire genomic *MYB101* region extending to adjacent upstream and downstream genes. The 5' and 3' UTRs are indicated by green and red boxes respectively. The coding region is indicated by a yellow arrow. Black boxes represent introns and the purple box indicates the miR159 target site. Figure is not to scale. The native *MYB101* target site sequence is shown on top of miR159a sequence and the mutated target site sequence is shown below, with mutated sites indicated in bold. The protein sequences of MYB101 and mMYB101 were identical.

After transformation into *Arabidopsis*, the phenotypes of multiple independent lines of transgenic plants were assessed. For both *MYB101* and *mMYB101,* the majority of lines appeared identical to wild-type (Fig 5.6 A, D, G), however some lines from both *MYB101* (9/21) and *mMYB101* (3/12) constructs produced upwardly curling leaves in the early stages of growth (Fig 5.7). The fact such phenotypes were produced in both *MYB101* and *mMYB101* lines indicates that the leaf curl phenotype is not solely determined by whether the miR159 target site is wild-type or mutated.

Flowers of *MYB101* and *mMYB101* plants from both wild-type looking and curly leaf transgenic lines were examined. In all cases flowers, anthers and pollen were indistinguishable from wild-type (Fig 5.6 B, C, E, F, H, I). qRT-PCR analysis of total *MYB101* transcript (endogenous plus transgenic *MYB101*) in inflorescences confirmed that the transgenic lines had increased levels of *MYB101* transcripts (Fig 5.6 J). Therefore rendering the miR159 target site in *MYB101* resistant to miRNA-regulation and/or over-expression of *MYB101*, has no obvious phenotypical consequences for anther or pollen development.

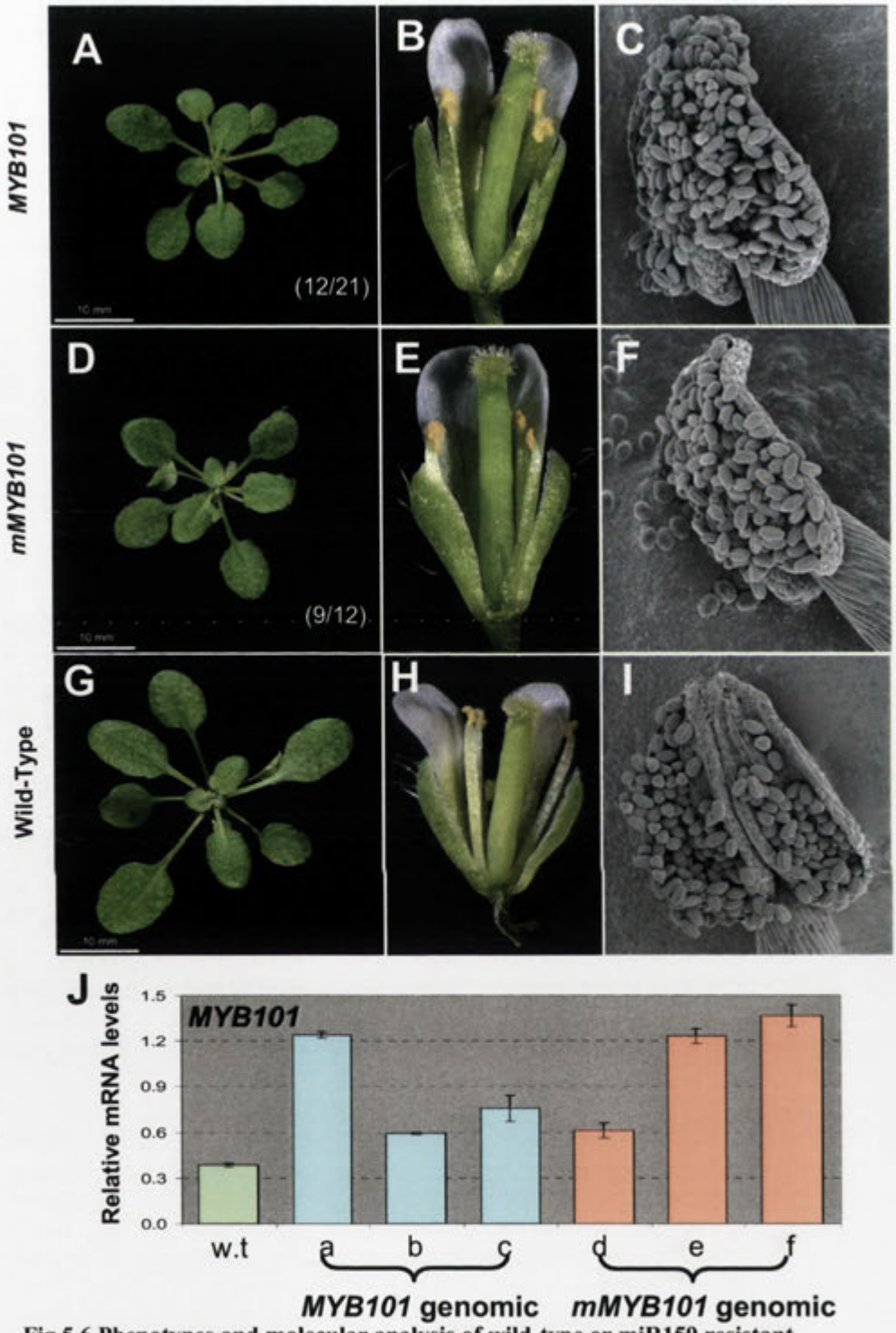


Fig 5.6 Phenotypes and molecular analysis of wild-type or miR159 resistant genomic *MYB101* transgenic plants.

Genomic *MYB101* and *mMYB101* constructs were transformed into *Arabidopsis. (*A, D) Aerial views of rosettes that show a wild-type (G) phenotype, numbers in brackets indicate the proportion of lines showing the wild-type phenotype. Flowers (B, E) and pollen (C, F) of all transgenics examined were normal compared to wild-type (H, I). (J) qRT-PCR analysis of *MYB101* expression in inflorescences of transgenic lines. Wild-type and three independent lines of *MYB101* (lines a,b,c) and *mMYB101* (d,e,f) were assayed. At least six biological replicates were used for each RNA sample. Values represent mRNA levels relative to *Cyclophillin*. Error bars represent SEM.

qRT-PCR analysis of *MYB101* transcript levels in rosettes of *MYB101* and *mMYB101* lines that displayed curly leaf phenotypes was carried out. It was found that steady-state levels of total *MYB101* (endogenous and transgenic *MYB101*) were significantly higher than wild-type (Fig 5.7). This demonstrates the *MYB101* transgene was being transcribed ectopically rather than representing native *MYB101* expression, and high levels of *MYB101* transcript can accumulate in these tissues. Exceptionally high total *MYB101* levels in a *mMYB101* line that displays leaf curling (Fig 5.7) are consistent with the mutated miR159 target site providing resistance to miR159 regulation, thus allowing excessive transcript accumulation for this line.

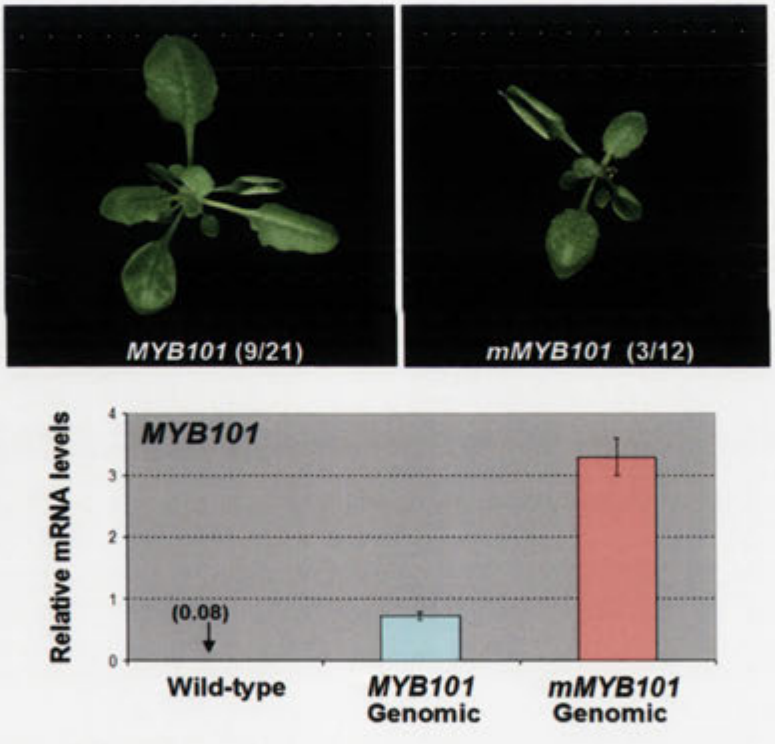


Fig 5.7 Rosette phenotypes and molecular analysis of *MYB101/mMYB101* over-expressing plants. Aerial views of transgenic *MYB101* and *mMYB101* rosettes displaying leaf curling, with numbers in brackets indicating the frequency of the leaf curl phenotype. *MYB101* transcripts from both *MYB101* and *mMYB101* leaf curl rosettes were assayed by qRT-PCR. The number in brackets shows the level of *MYB101* in wild-type rosettes. At least six biological replicates were used for each RNA sample. Values represent mRNA levels relative to *Cyclophillin*. Error bars represent SEM.

5.2.4 miR159 does not influence the *MYB101* expression domain in anthers

Although the 5'-RACE data suggest that *MYB101* could be miRNA-regulated, *MYB101* transcript levels are not dramatically different between wild-type and *mir159ab*, even in inflorescence tissue, where *MYB101* is predominantly expressed (Chapter 4, Fig 4.1). However these previous analyses have only examined regulation at the gross tissue level, and they do not take into account possible translational regulatory mechanisms, or the possibility of miR159c regulation. To take these factors into account, constructs that contained *MYB101* genomic sequence fused in frame to GUS were transformed into *Arabidopsis*, with either the miR159 binding site intact (*MYB101:GUS*) or mutated (*mMYB101:GUS*) (Fig 5.8). This would allow any miRNA-regulation of *MYB101* expression to be visualised, similar to the *MYB33:GUS* versus *mMYB33:GUS* analysis (Millar and Gubler., 2005).

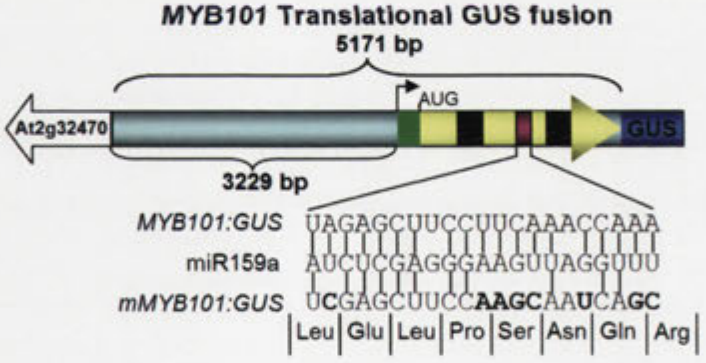


Fig 5.8 *MYB101:GUS/mMYB101:GUS* constructs used to transform *Arabidopsis*

5171 bp of genomic sequence was amplified from *Arabidopsis* and included the entire genomic *MYB101* region extending to the adjacent upstream gene. The *MYB101* stop codon was removed and replaced with GUS coding sequences (dark blue box). The *MYB101* 5' UTR is indicated by the green box. The *MYB101* coding region is indicated by the yellow arrow. Black boxes represent introns and the purple box indicates the miR159 target site. Figure is not to scale. The native *MYB101* target site sequence is shown on top of miR159a sequence and the mutated target site sequence is shown below, with mutated bases indicated in bold. The protein sequences of *MYB101:GUS* and *mMYB101:GUS* were identical.

However, unlike the previous *MYB33:GUS* experiment (Millar and Gubler, 2005), primary transgenic lines of both *MYB101:GUS* and *mMYB101:GUS* displayed wild-type (Fig 5.9) or mutant (leaf curling) phenotypes, although flowers and pollen appeared normal in all lines. This implies the MYB101:GUS fusion protein has biological activity and is influencing development. As plants with mutant phenotypes were likely to be strongly over/mis-expressing the transgene, the analyses of these plants will be described in the next section. Only *MYB101:GUS* and *mMYB101:GUS* plants that had wild-type rosette phenotypes (Fig 5.9), and therefore more likely to have expression patterns and levels reflecting *in vivo MYB101* were used in the analysis.

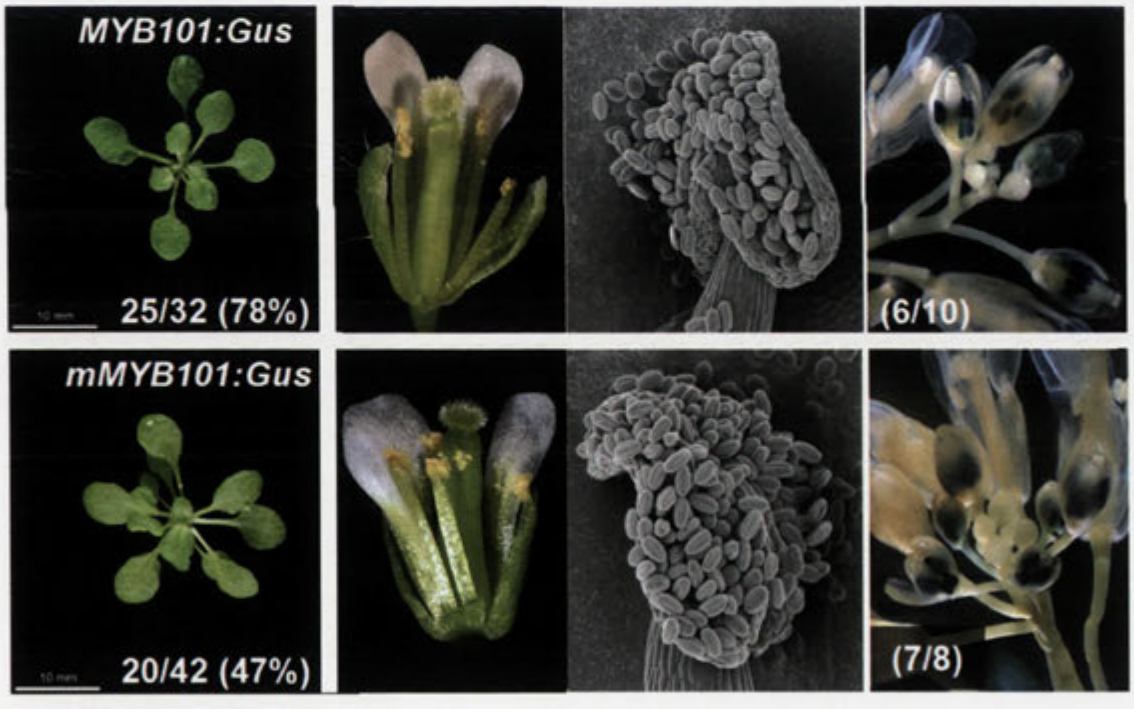


Fig 5.9 Analysis of *MYB101:GUS/mMYB101:GUS* transgenic plants
The phenotypes of transgenic lines are shown. Numbers below rosettes indicate the proportion of lines that had wild-type appearing rosettes. Flowers and pollen were normal in all lines. For GUS staining, numbers inside brackets indicate the proportion of wild-type appearing lines stained that showed the anther specific expression pattern.

The GUS expression pattern from the majority of these lines showed expression in anthers (Fig 5.9). This is consistent with the pattern seen for a *proMYB101*:GUS promoter only construct (Fig 5.1) and with Affymetrix data showing *MYB101* is overwhelmingly expressed in anthers and pollen (Hruz, 2008). Although at the gross level, no clear evidence of an expanded *MYB101* expression domain could be observed in *mMYB101:GUS* when compared to *MYB101:GUS* plants, it is possible that more subtle regulation occurs in discrete anther cell layers. Therefore, transverse sections of GUS stained anthers were examined to determine if the temporal or spatial expression domain of the *mMYB101:GUS* transgene differed to that of *MYB101:GUS*. Cross-sections were prepared from GUS stained *MYB101:GUS* and *mMYB101:GUS* lines that had normal rosette phenotypes, and compared with *proMYB101:GUS* anther sections. GUS staining in all sections appeared similar, with abundant GUS crystals appearing in the tapetum (Figure 5.11). In all three lines, GUS crystals were also present in other cell-types, including the developing micro-spores, connective and other anther cell layers. However there was no obvious difference between staining in *MYB101:GUS* anthers, compared to the two miR159-insensitive lines, *proMYB101:GUS* and *mMYB101:GUS*. This indicated that miR159 does not delineate expression of *MYB101* in anthers, where its expression is predominantly regulated through transcriptional means.

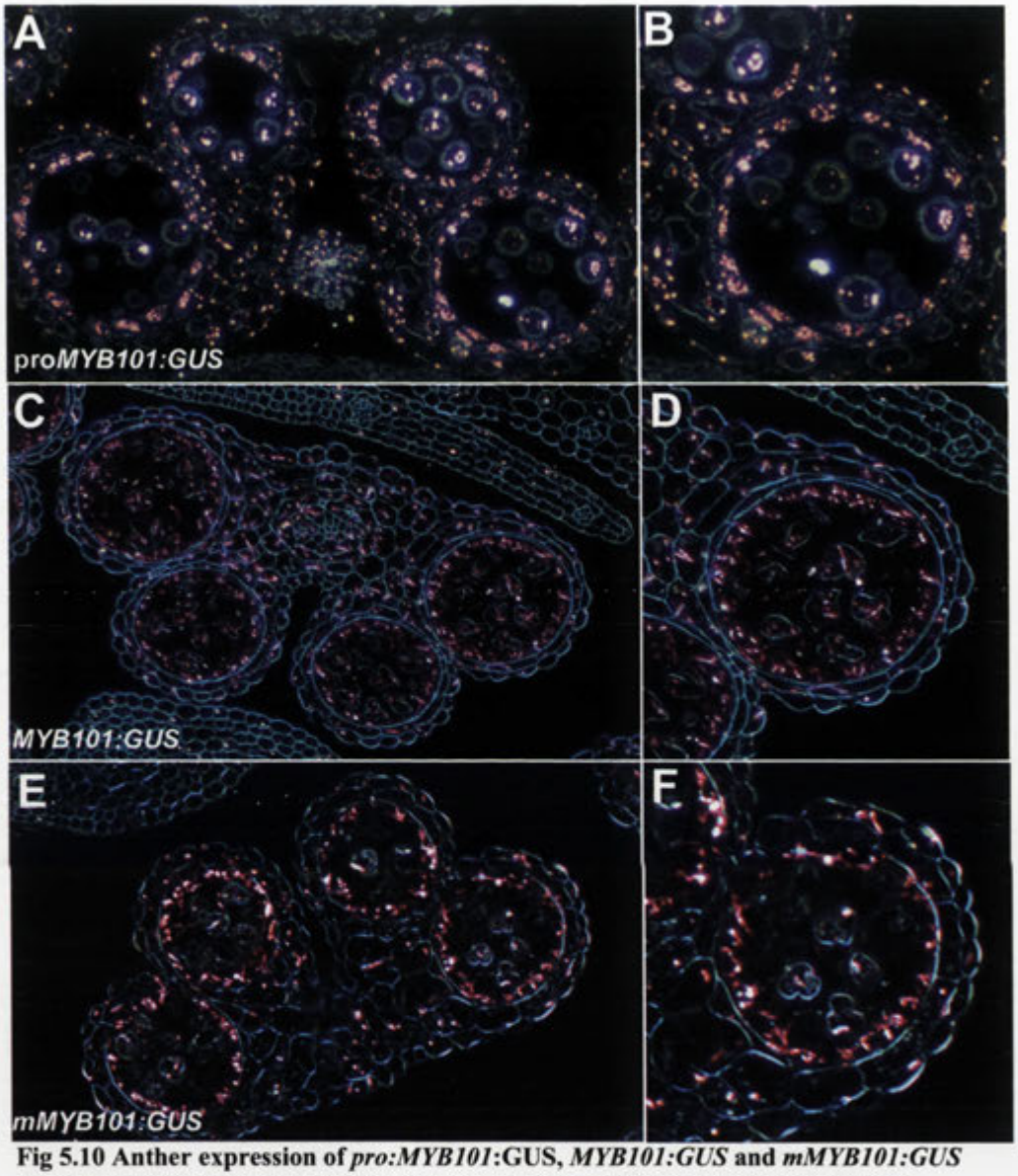


Fig 5.10 Anther expression of *pro:MYB101*:GUS, *MYB101:GUS* and *mMYB101:GUS*
Inflorescences were stained overnight and embedded in paraffin. Cross-sections were examined by dark field microscopy. Whole anthers are shown on the left panels with detail of individual locules on right. GUS staining is shown by pink crystals in pro:*MYB101*:GUS (A,B), *MYB101:GUS* (C,D) and *mMYB101:GUS* (E,F). Images A and B were supplied by Anthony Millar.

5.2.5 *MYB101* is not strongly silenced by miR159

As previously mentioned, unlike the analogous experiment with *MYB33:GUS* and *mMYB33:GUS* (Millar and Gubler, 2005), where neither construct resulted in developmental abnormalities, expression of both the *MYB101:GUS* (7/32) and *mMYB101:GUS* (21/42) transgenes could result in developmental phenotypes. Furthermore the *mMYB101:GUS* transgenic lines displayed noticeably more severe leaf curling than the *MYB101:GUS* lines (Fig 5.11 A). As *mMYB101:GUS* plants displayed a much higher frequency and severity of this phenotype again demonstrates that like the *MYB101* transgene, the *MYB101:GUS* transgene can be miRNA regulated when expressed in the expression domain of miR159a/b.

These phenotypes had similarities to the *mir159ab* and *mMYB33* phenotypes, suggesting that they are arising due to MYB activity in rosette tissues due to strong *MYB101* over-expression. This was confirmed by qRT-PCR analysis of inflorescence *MYB101* levels, where both *MYB101:GUS* and *mMYB101:GUS* lines had higher *MYB101* levels than wild-type (Fig 5.11 B). Furthermore a *mMYB101:GUS* line that showed severe leaf curling and expanded GUS activity had higher rosette *MYB101* transcript levels than a *mMYB101:GUS* line that displayed a wild-type rosette phenotype and anther specific staining (Fig 5.11 C). However similar to *MYB101* transgenic lines, the fact that *MYB101:GUS* lines can display a mutant phenotype at all suggests that the repression of *MYB101* by miR159 is not absolute. This contrasts to transgenic *MYB33* over-expression lines that never displayed a phenotype (Millar and Gubler, 2005). This suggests that the repression of *MYB101* expression by miR159 is much weaker than the miR159 repression of *MYB33*.

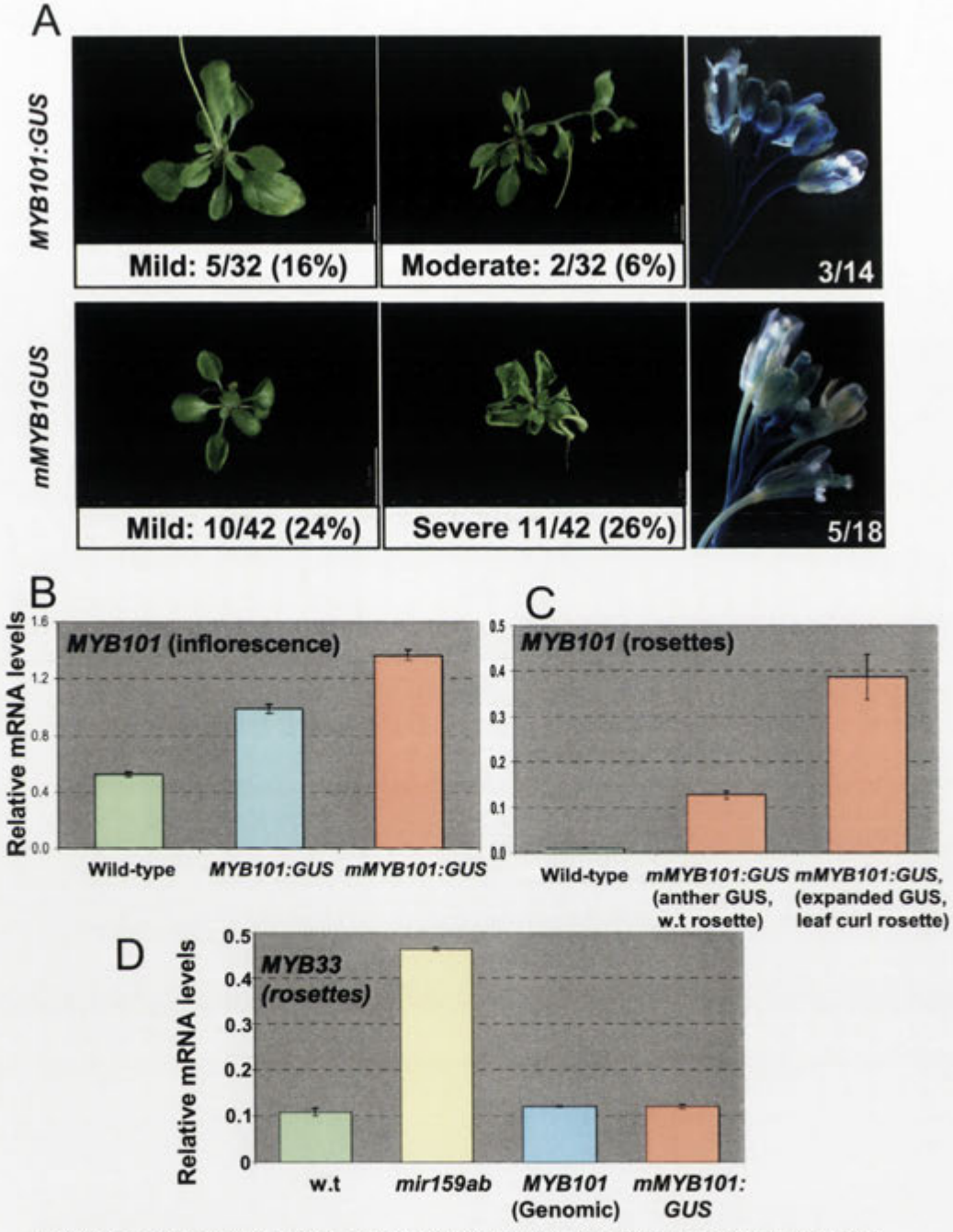


Fig 5.11 Phenotypes and molecular analysis of *MYB101:GUS* and *mMYB101:GUS* overexpressing plants

(A) For transgenic lines that showed leaf curling, phenotypes were classified as mild, moderate or severe. The proportion of each phenotype to total lines is indicated for each phenotype. *MYB101:GUS* lines produced no severe leaf curl phenotypes, and only one *mMYB101:GUS* line produced moderate leaf curl (not shown). GUS patterns representative of GUS expanded *MYB101:GUS/mMYB101:GUS* lines are shown, and the proportion of each line showing the representative pattern to the number of stained lines is indicated. (B) *MYB101* transcript was measured from the inflorescence of *MYB101:GUS* and *mMYB101:GUS* leaf curl plants that showed expanded GUS expression. (C) *MYB101* transcript was also measured from the rosettes of *mMYB101:GUS* lines that had wild-type rosettes and anther specific GUS staining, or severe leaf curl and expanded GUS staining. (D) *MYB33* was measured in the rosettes of wild-type, *mir159ab*, *MYB101* (genomic) and *mMYB101:GUS* lines. At least six biological replicates were used for each RNA sample. Values represent mRNA levels relative to *Cyclophillin*. Error bars represent SEM.

Consistent with the over-expression of *MYB101* in both *MYB101:GUS* and *mMYB101:GUS* lines that showed leaf curling, most assayed leaf curling lines also showed GUS staining extending beyond anthers (Figure 5.11 A). These lines generally showed expansion of GUS expression into different tissues; stems, sepals, and sometimes the stigma. However even in these severe leaf curl lines, the over-expression of *MYB101:GUS* does not appear to affect anther or pollen development, as inflorescences and pollen appeared normal (Fig 5.9). Finally, to dismiss the possibility that the leaf curl phenotypes were a consequence of elevated *MYB33* levels (perhaps due to the sequestration of miR159 by the *MYB101* transgene), both *MYB101* and *MYB101:GUS* lines were assayed for *MYB33* transcript (Fig 5.11 D). However *MYB33* levels were unchanged in all transgenic lines assayed, in contrast to *mir159ab* where *MYB33* levels are considerably higher. Therefore the curly leaf phenotype seen in *MYB101* transgenic plants is not caused by increases in *MYB33* transcript, and given that *MYB33* is at wild-type levels in these lines, this demonstrates miR159 function is uncompromised in these transgenic plants.

5.3 Discussion

5.3.1 miR159 regulation of *MYB101* has no apparent *in vivo* relevance

The 5'-RACE recovery of miRNA-guided cleaved mRNA targets has been considered the gold standard for determining if a particular mRNA is an authentic miRNA target (German et al., 2008; Llave et al., 2002). Here it has been shown that even the proven cleavage of an mRNA target gives no indication if a miRNA-target relationship has *in vivo* significance. Similarly, analysis of phenotypical changes resulting from disruption of miRNA binding sites in putative mRNA targets has been employed to describe the importance of miRNA-mediated regulation. However, the results here show that transgenic over-expression can potentially misrepresent the importance of miRNA regulation for a particular target, even when transcribed under its native promoter using extensive flanking regions extending to adjacent genes.

Specifically, it has been shown that *Arabidopsis* miR159 is not critical for either delineating the expression domain of *MYB101,* or directly regulating its expression level. There are several lines of evidence supporting this claim. Firstly, the wild-type appearance of anthers and pollen in all *MYB101* and *mMYB101* transgenic plants underscores the independence of *MYB101* from miR159 regulation. Additionally, 5'-RACE demonstrated that relative to *MYB33*, cleavage of *MYB101* is extremely rare. This is supported by the lack of any *MYB101* cleavage products found in three different degradome deep sequencing experiments (Addo-Quaye et al., 2008; German et al., 2008; Gregory et al., 2008). Furthermore, when aberrant phenotypes were observed, they were found to occur in both *MYB101* and *mMYB101* plants, and appeared in rosettes where *MYB101* is normally transcribed at extremely low levels. Finally qRT-PCR analysis of these plants provided a molecular explanation for these phenotypes,

where curly leaf *MYB101/mMYB101* plants were found to be over-expressing *MYB101* in rosettes 5-40 fold. Most remarkably, despite bioinformatic predictions and 5'-RACE confirmation that *MYB101* is miR159 regulated, this gene is clearly not able to be as effectively silenced by miR159 as *MYB33*. This is implicitly demonstrated by comparison of leaf curl lines ectopically expressing *MYB101* with *35S:MYB33* or genomic *MYB33* lines, which produced no phenotype additional to wild-type (Millar and Gubler, 2005; Palatnik et al., 2003). The implications of this result will be discussed later.

5.3.2 Recovery of 5'-RACE cleavage products may misrepresent the importance of miRNA regulation for some mRNAs.

The results of 5'-RACE for *GAMYB-like* targets further demonstrated the discrete specificity of miR159 regulation for only *MYB33/MYB65* among other potential targets. That is, while *MYB33* cleavage products were relatively easy to recover, cleavage products for the other *GAMYB-like* genes were considerably rarer or non-existent. This was reinforced during the course of this study, where although two reports showed *MYB101* can be cleaved (Alves-Junior et al., 2009; Reyes and Chua, 2007), both showed only 50% (4/8) and 29% (4/14) of cloned fragments matched the site of miR159-mediated *MYB101* cleavage respectively, further demonstrating that miR159-mediated *MYB101* cleavage fragments are far less easy to detect than *MYB33* fragments. Further recent 5'-RACE efforts augmented by deep sequencing have failed to find any cleaved *MYB101* transcripts (Addo-Quaye et al., 2008; German et al., 2008; Gregory et al., 2008), again demonstrating that miR159 cleavage of *MYB101* is a rare occurrence. However a major weakness in cleavage assays is their inability to discern possible translational repression. Yet this appears not to occur for *MYB101,* because

expression patterns for *MYB101*:GUS and *mMYB101*:GUS were indistinguishable from *proMYB101:GUS* in the majority of wild-type appearing plants.

The fact that *GAMYB-like* cleavage products were even rarer in *mir159ab* would suggest that miR159a and miR159b are the major cleavage regulators of the *GAMYB-like* genes. Because *MIR159a/MIR159b* and *MYB101/MYB81* appear to be expressed in mutually exclusive expression domains, the *MYB101* and *MYB81* cleavage products may arise from aberrant *MYB101/MYB81* transcription outside their normal domain, where miR159a and miR159b are expressed. Such aberrant *GAMYB-like* transcription would appear to be rare in this scenario for two reasons. Firstly as described above, the fact that only nested PCR can recover with low efficiency *MYB101* cleavage products suggests that cleavable *GAMYB-like* transcripts are rare. Secondly the *mir159ab/myb33/myb65* mutant has a wild-type phenotype outside of anthers, demonstrating that any aberrant *GAMYB-like* transcripts must be sufficiently low as to produce no phenotypical consequence when devoid of miR159a/b regulation.

The converse possibility, that *MIR159a/MIR159b* is occasionally expressed in anthers/pollen may also apply. Indeed two recent studies have provided conflicting evidence both for and against this possibility, where in one case miR159a and miR159b was undetectable in pollen (Chambers and Shuai, 2009), but in another using deep sequencing, miR159a and miR159b appeared to be highly abundant in this tissue (Slotkin et al., 2009). The results of the latter study are inconsistent with multiple experiments showing expression of miR159a under 35S promoter sequences leads to anther defects (Achard et al., 2004; Schwab et al., 2005), and evidence from two independent reporter constructs (*MIR159a:GUS* and *MIR159b:GUS*) showing

MIR159a and *MIR159b* are widely expressed in most tissues except anthers/pollen (Allen et al., 2007).

5.3.3 Ectopic expression of transgenic *MYB101* demonstrates a lower efficiency of miR159 regulation compared to *MYB33*

Transgenic expression of *MYB101* has demonstrated a surprising facet of miR159 regulation of *GAMYB* genes; there is a distinct difference in the sensitivity of *MYB101* to miR159 regulation compared to *MYB33*. Given previous evidence, it would be reasonable to assume that *MYB101* overexpressed in the domain of miR159 would be down-regulated comparably to *MYB33*. This is based firstly on the proven cleavage of *MYB101* by miR159. Secondly the fact that *MYB33* is effectively down-regulated in the domain of miR159 indicates miR159 has strong silencing ability - this is best exemplified by the absence of any phenotype in transgenic *35S:MYB33* and *MYB33* plants (Millar and Gubler, 2005; Palatnik et al., 2003). Thirdly, even when only one copy of miR159b is present in a *mir159a/MIR159B/mir159b* mutant, miR159 is still able to down-regulate *MYB33* and *MYB65* to the extent that no phenotype is apparent- this indicates miR159 is in excess, and at native expression levels would have significant residual ability to down-regulate excess *GAMYB*. However despite these assumptions, the experimental evidence shows miR159 is unable to down regulate *MYB101* to the same extent it can *MYB33*.

It is interesting that despite containing all genomic sequences extending to adjacent genes both upstream and downstream of *MYB101*, the *MYB101* genomic construct did not always faithfully recapitulate native *MYB101* expression. Fortuitously however, the ectopic expression of *MYB101* in the domain of miR159a/b revealed *MYB101* can

function comparably to *MYB33*, as the leaf curl phenotypes occasionally produced in these lines were similar to, though less severe than the *mir159ab* mutant. Notably, *MYB101* and *MYB33* are both strongly expressed in the tapetum (Fig 5.7, Millar and Gubler 2005), and the similarity in the phenotypes produced by their overexpression suggests they operate in the same pathways. However these genes clearly have different regulatory options despite them both containing miR159 sites; *MYB33* appears broadly transcribed but confined to anthers by miR159 regulation, whereas *MYB101* has a very discrete transcriptional domain that is not delineated by miR159.

There is evidence that other RACE validated targets of miR159 may also be less sensitive to miR159 regulation; *DUO1*, when over-expressed with its native miR159 target site, was found to produce aberrant phenotypes (Palatnik et al., 2007). Therefore although the functional specificity of miR159 for *MYB33/MYB65* is due to miR159 transcriptional domain overlap with *MYB33/MYB65*, and non-overlap with other anther/pollen *GAMYBs*, intrinsic differences between miR159 targets may provide a further level of efficiency in regulation of *GAMYB* genes by miR159.

Further experiments would be required to determine the nature of this differential regulation of *GAMYB-like* and *MYB* genes by miR159. Nevertheless this result reveals that different 5'-RACE verified targets can be down-regulated to different extents. Given that the *GAMYB-like* miR159 target sites are highly similar but not identical (except for *MYB33/MYB65*), minor regulatory differences would seem plausible, and may have biological implications. However, the fact that seemingly minor differences in target sites (such as occur between *MYB33* and *MYB101*) can lead to dramatically different regulatory outcomes is remarkable- particularly considering that *MYB101* has

less mismatches to miR159 than *MYB33*. Intriguingly, a mismatch between *MYB101* and miR159 occurs at the same position (six nt with respect to the 5' end of the miRNA) as the gain-of-function *Lanceolate* mutation in tomato which disrupts miR319 regulation of this mutant allele (Ori et al., 2007). This suggests mismatches at this position may render targets more resistant to miRNA regulation. However other determinants such as secondary structure around the target site, and target interacting proteins have all been shown in some cases to affect target down-regulation (Kedde et al., 2007; Kertesz et al., 2007), and any of these factors could conceivably influence miR159 regulation of different *GAMYB*s. Additionally the free energy difference between a miRNA and its target remains the only empirical measure of miRNA:target affinity (Doench and Sharp, 2004), and although this measure does not always distinguish genuine miRNA targets, for *MYB101* the free energy is less favourable than for *MYB33*.

Given that *MYB101* contains a conserved miR159 site, the notion that its regulation by miR159 has no *in vivo* importance may seem counterintuitive. Yet nature is replete with non-essential remnants of ancient biological systems (the human appendix being a classic example), and it appears that miR159 regulation of *MYB101* may represent such a case. This again highlights an emergent theme in plant and animal miRNA studies, where although individual miRNAs may be expected to target many different mRNAs, there is a smaller subset of biologically relevant targets than predicted by bioinformatics or shown by overexpression studies. Therefore at least *MYB101* falls into this category of inconsequential targets of miR159. However there are several other verified (*MYB81*, *DUO1*), and potential targets of miR159, and given they appear not to contribute to the *miR159ab* phenotype, it remains possible they may be subject to miR159c regulation. This will be investigated in the following chapter.

Chapter 6

Functional analysis of *Arabidopsis* miR159c

The problem with Rob and miR159c
is he's 1,000,000 years too late
-Anonymous post lab-chat comment

6.1 Introduction

The previous chapters have revealed the discrete specificity of the miR159a/miR159b: *MYB33/MYB65* regulatory module. A broad corollary of this result is miR159a/miR159b regulation of *MYB101* and possibly other potential targets is not important for *Arabidopsis* development. However, the previous chapter's main focus on *MYB101*, one of several potential targets, left unresolved the possibility that miR159c may play a role in their regulation.

This notion was given greater impetus during the course of this study, when a *MIR159c:GUS* transgene (Fig 6.1) was shown to be expressed in inflorescences, specifically in anthers (Junyan Li - personal communication Figure 6.1) remarkably similar to *MYB101:GUS* (chapter 5). Thus the *MIR159c* expression domain appears reciprocal to that of *MIR159a/MIR159b*, but may overlap with *MYB101*, and possibly other miR159 targets that are predominantly transcribed in anthers and pollen. Furthermore 5'-RACE miR159 guided cleavage products for both *MYB33* and *MYB81* were recovered in the *mir159ab* mutant (Chapter 5), and although these products could represent residual miR159a or miR159b cleavage, it is possible that miR159c could be responsible for these cleavage products.

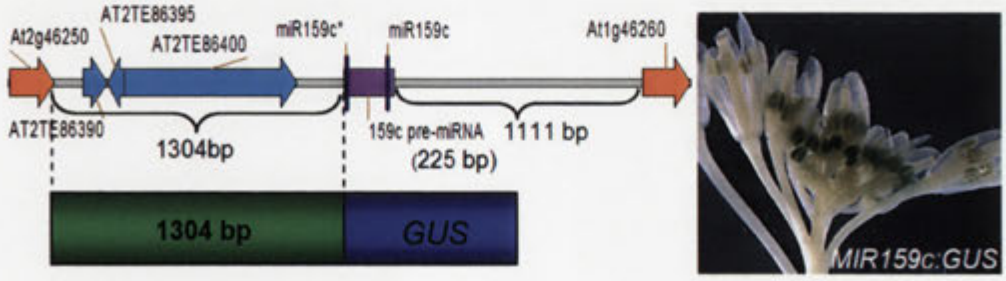


Fig 6.1 Expression of a *MIR159c:GUS* construct in *Arabidopsis* inflorescences
The genomic context of miR159c is shown to scale with the corresponding region below used for creation of a GUS fusion construct. All genomic sequences upstream of predicted pre-miR159c sequence to the next upstream gene were used. Multiple transgenic lines showed GUS staining in anthers (photo courtesy of Junyan Li)

Chapter 3 demonstrated that a T-DNA knockout of *MIR159c* did not lead to any rosette phenotype, or any additional phenotypes when introduced into the *mir159ab* mutant. One explanation for these results is low abundance of miR159c prevents it from contributing to target regulation. The scarcity of miR159c has been partially attributed to inefficiency of miR159c processing. This is because Palatnik et al, (2007), using northern blotting, were unable to detect overexpression of mature miR159c when using the 35S promoter immediately upstream of miR159c stem-loop, despite being able to detect expression of the transgenic stem-loop sequence. In contrast, overexpression of miR159a was detectable by northern blotting and led to anther defects in *Arabidopsis*.

However this work might have been unable to determine that miR159c could be overexpressed, since the sensitivity of northern blotting for miR159 members may not be sufficient to detect minor increases in miR159c. It is also conceivable that additional sequences both upstream and downstream of the *MIR159c* stem-loop may be required for efficient processing of miR159c, and this area has been little explored. This possibility was supported in this study, where T-DNA insertions both upstream and downstream of the *MIR159a* stem-loop region were shown to effect processing of miR159a (Chapter 3).

Furthermore the notion that relatively small miRNA levels can produce noticeable impacts has been demonstrated in one of the few other plant miRNA loss-of-function mutants characterised to date; in the miR164 family, miR164c comprises only 0.4% of total miR164 abundance (Backman et al., 2008), yet a *mir164c* loss of function mutant shows floral defects (Baker et al., 2005). Therefore it is possible that despite low abundance, miR159c may play a specialised and/or subtle role. Additionally, expression

of *MIR159c* could conceivably increase under different environmental circumstances, as has been demonstrated for other stress inducible miRNAs (Ding et al., 2009; Jian et al., 2009). Finally, functional analysis of the *mir159c* loss-of-function mutant would complete characterisation of the entire miR159 family in *Arabidopsis*, providing an overview of the regulatory landscape encompassed by *Arabidopsis* miR159.

Therefore the aims of this chapter are:

1) Determine if miR159c expression overlaps with anther transcribed miR159 targets.
2) Determine whether there are anther/pollen defects in *mir159c*.
3) Determine if potential targets are deregulated in *mir159c*
4) Over-express *MIR159c*.

6.2 Results

6.2.1 *MIR159c* is predominantly expressed in the tapetum

GUS staining of inflorescences from a *MIR159c:GUS* construct revealed anther specific staining (Fig 6.1) similar to *MYB101:GUS*, suggesting that their expression domains overlap. Therefore to precisely determine if *MIR159c* was transcribed in the same domain of *MYB101* or different cell layers, a more detailed examination was undertaken. Transverse sections of GUS stained *MIR159c:GUS* anthers were examined by dark-field microscopy, and showed staining in the tapetum of developing anthers (Fig 6.2). This staining pattern appeared similar to *MYB101:GUS*, (Chapter 5) but generally weaker. This confirmed that *MIR159c* is expressed in the same cell types of at least one of the anther/pollen predominant *GAMYB-like* targets. It is interesting that *MIR159c* appears to be expressed in the same cell layer as *MYB101*, despite the presence of a transposable element that may reside in the transcribed region of *MIR159c*, possibly influencing its expression. This may account for the low level of pri-miR159c as measured by qRT-PCR (Chapter 3).

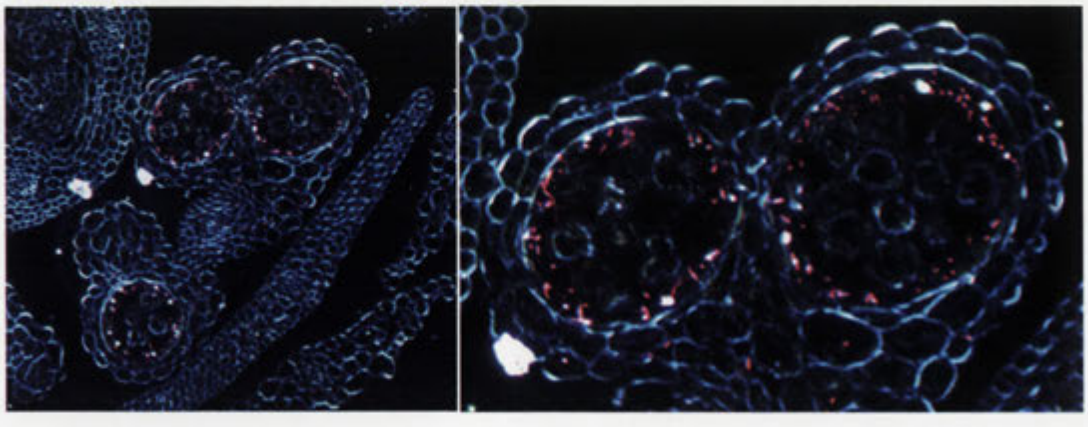


Fig 6.2 Analysis of *MIR159c*:GUS expression in anthers
Dark field microscopy of transverse sections of *MIR159c:GUS* stained inflorescence. Detail of individual locules is shown at right. GUS staining is visualised as pink crystals.

6.2.2 The *GAMYB-like* genes show no transcriptional deregulation and pollen development is normal in *mir159c.*

To determine if there was any phenotypical consequence of miR159c loss, anthers were examined in the *mir159c* mutant. However the *mir159c* mutant was generated in a *quartet1* (*qrt1*) background, where pollen grains form tetrads (Figure 6.3) (Preuss et al., 1994). As this could be masking any defects in pollen development caused by the lack of miR159c, the *mir159c/qrt1* mutant was backcrossed to wild-type (Columbia) plants. *mir159c/QRT1* plants were obtained and SEM examination of their anthers and pollen revealed no difference in morphology compared to wild-type (Fig 6.4 A-D). Thus if there is any consequence to miR159c loss in anthers, where *MIR159c* appears to be transcribed, the biological consequences are too subtle to be noticed by these methods.

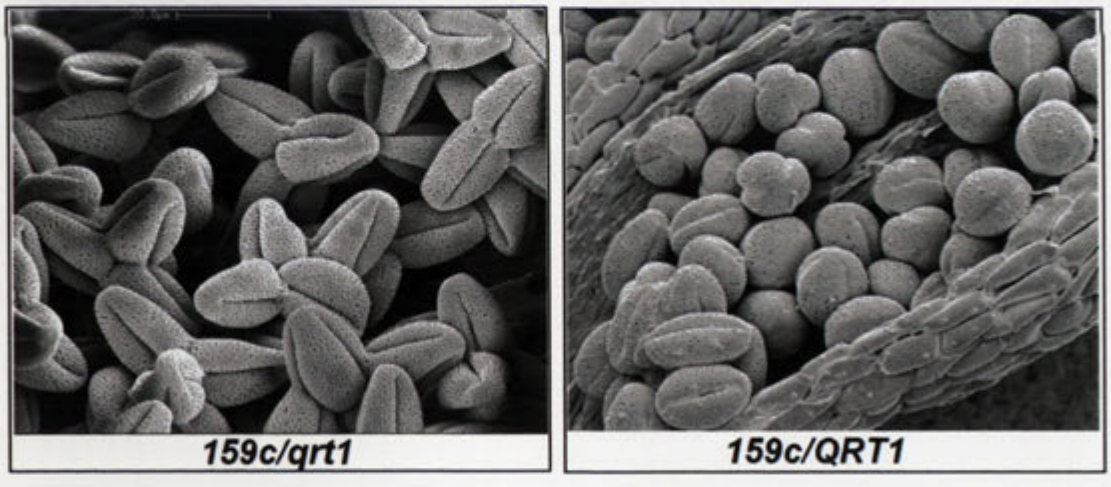


Fig 6.3: Removal of *qrt1* from *mir159c*
Homozygous *mir159c/qrt1* pollen forms tetrads (left). Homozygous *mir159c/QRT1* plants no longer show the tetrad phenotype.

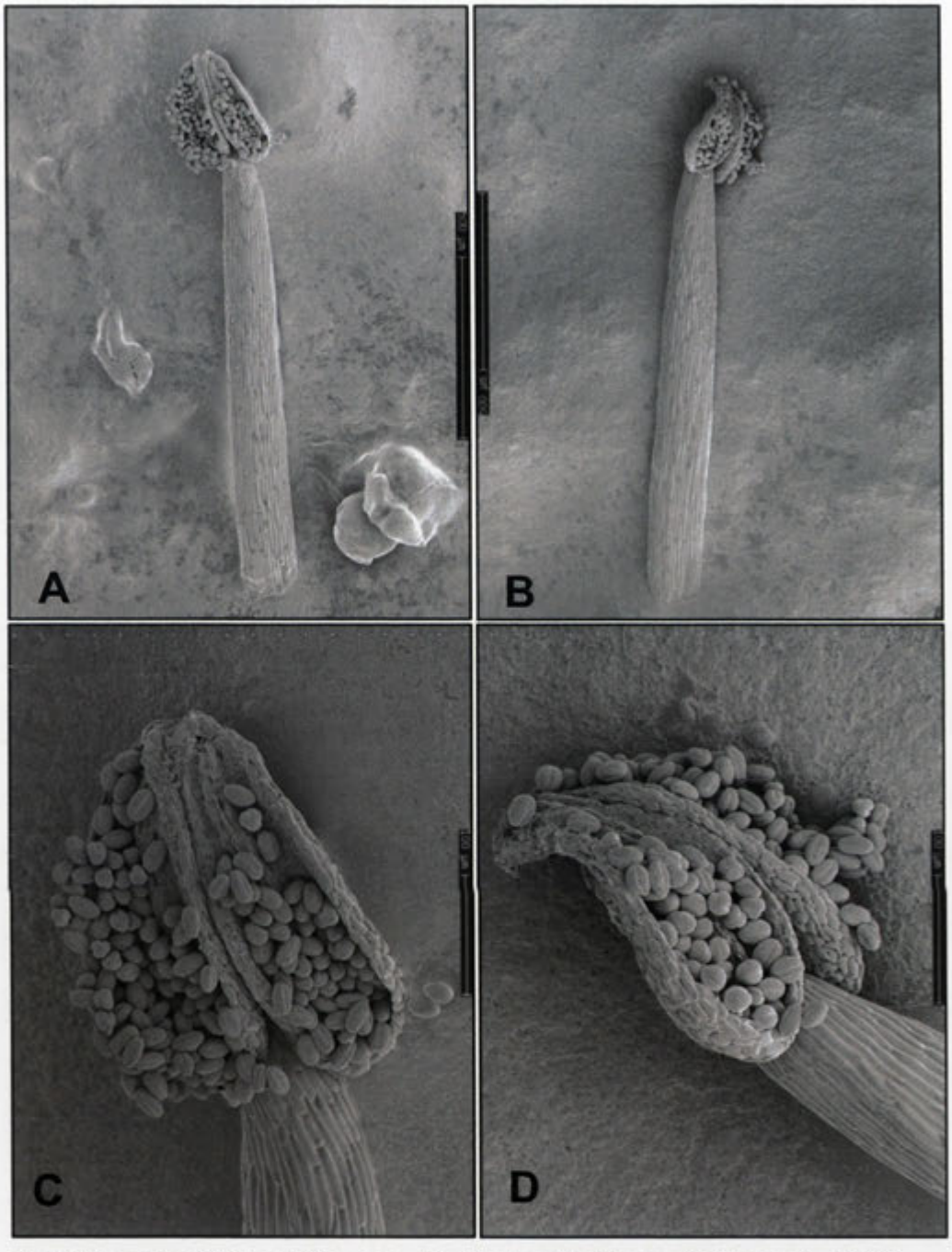


Fig 6.4 Scanning Electron Microscopy of anthers and pollen in wild-type and *mir159c*
Isolated anthers from mature inflorescences were examined by SEM microscopy. (A) wild-type stamen/anther (B) *mir159c* stamen/anther (C) detail of wild-type anther/pollen (D) detail of *mir159c* anther/pollen.

Next potential mRNA targets of miR159c were examined for transcript deregulation. In addition to *DUO1* and the *GAMYB-like* family of genes, miR159c is also predicted to regulate *TCP2* (Palatnik et al., 2007). In contrast to the deregulation of *MYB33/MYB65* seen in *mir159ab*, there were no major changes in transcript levels between *mir159c* and wild-type (Fig 6.5) in any of these genes. *MYB101*, which is the most strongly expressed *GAMYB-like* gene in inflorescence, had virtually identical steady-state mRNA levels compared to wild-type. *MYB81* expression was actually lower in *mir159c,* but not significantly so ($P < 0.05$, students T- test). Given the low expression levels shown for this gene, and the fact that *mir159c* inflorescences were indistinguishable from wild-type, it is unlikely this change has any biological significance. Although the possibility of translational repression by miR159c of these *GAMYB-like* targets remains, the fact that the *mir159c* mutant appears indistinguishable from wild-type would again argue that, if this was the case, its appears to have little biological impact, if any.

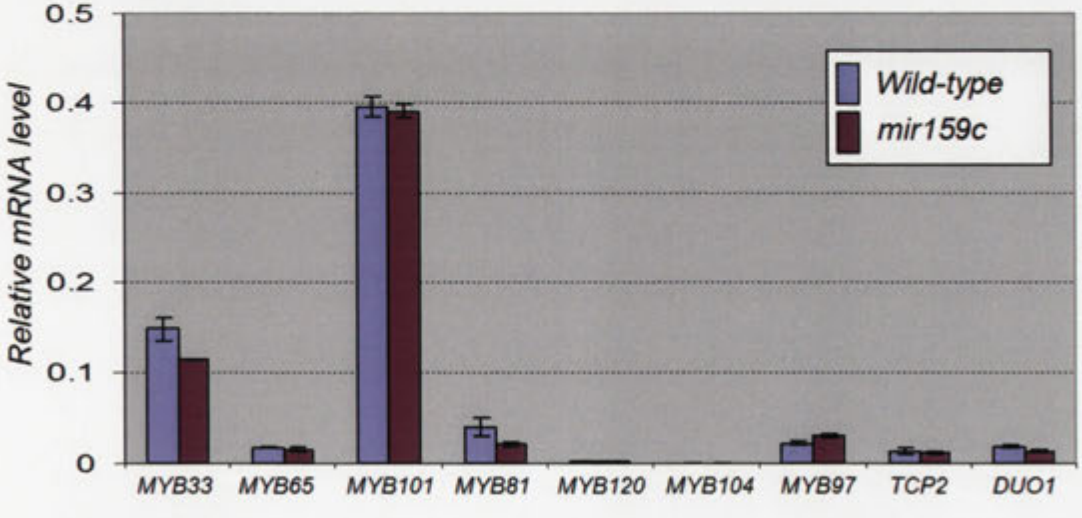


Fig 6.5 Analysis of miR159c target gene expression in *mir159c* qRT-PCR analysis miR159 target gene expression in wild-type (blue bars) and *mir159c* (purple bars) was carried out. Analysis was performed on RNA extracted from inflorescences. At least six biological replicates were used for each RNA sample. Values represent mRNA levels relative to *Cyclophillin*. Error bars represent SEM.

This lack of transcript deregulation was reflected in the levels of mature miR159c in the *mir159c* mutant. Close sequence similarity between miR159a and miR159b makes an accurate determination of miR159c levels problematic in wild-type by qSL-PCR (Chapter 3). Therefore, the *mir159abc* mutant was compared with *mir159ab*. The low level of miR159c as measured by qSL-PCR was marginally higher in the *mir159abc* mutant (Fig 6.6), but not significantly so ($P < 0.05$ students T-test). This demonstrates the level of miR159c measured in inflorescences is so low that even the highly sensitive qSL-PCR method is unable to accurately measure endogenous miR159c.

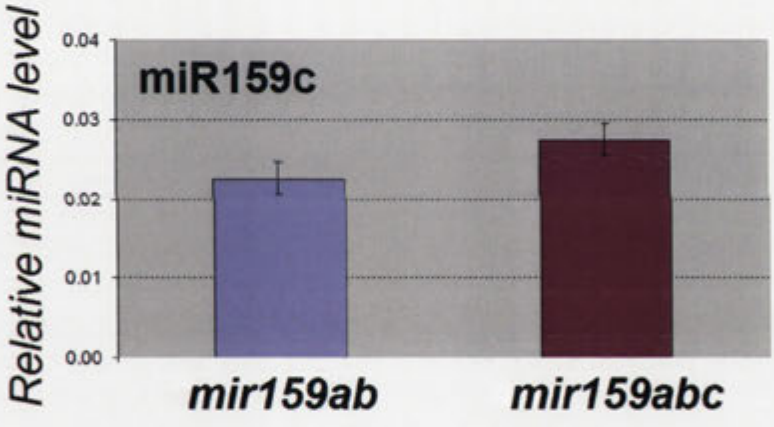


Fig 6.6 Quantification of miR159c levels. qSL-PCR of miR159c in *mi159ab* and *mir159abc*. Analysis was carried out on RNA extracted from inflorescences. At least six biological replicates were used for each RNA sample. Values represent miRNA levels relative to *sno101*. Error bars represent SEM.

6.2.3 A 35S:*MIR159c* transgene is unable to produce male sterility

It is remarkable that *MIR159c* appears to share an extremely discrete transcriptional domain with potential *GAMYB-like* targets. Very low levels of *MIR159c* expression and/or low processing efficiency of miR159c may account for why these targets appear not to be miR159c regulated and the *mir159c* mutant does not exhibit a mutant phenotype. However the possibility that under different conditions *MIR159c* may be more highly expressed and thus be able to significantly regulate the *GAMYB-like* or other target genes is worthy of consideration. To this end, it was determined to investigate whether miR159c could actually be over-expressed, or whether miR159c is incapable of being processed.

Therefore a *35S:MIR159c* construct was made that contained 525 bp of genomic sequence upstream of *MIR159c* stem-loop, and 485 downstream (Fig 6.7 A). This upstream sequence included 300 bp of the transposable element AT2TE86400. An RNA fold of this region showed the transgenic *MIR159c* stem-loop is predicted to fold into the same predicted structure as native *MIR159c* stem-loop (Fig 6.7 B, C). Therefore this construct was similar to the previously used *35S:MIR159c* construct of Palatnik et al., (2007). However this significantly longer construct also contained upstream and downstream regions of the native *MIR159c* sequence that may be important for production of miR159c. This construct was transformed into *Arabidopsis* and phenotypes were examined.

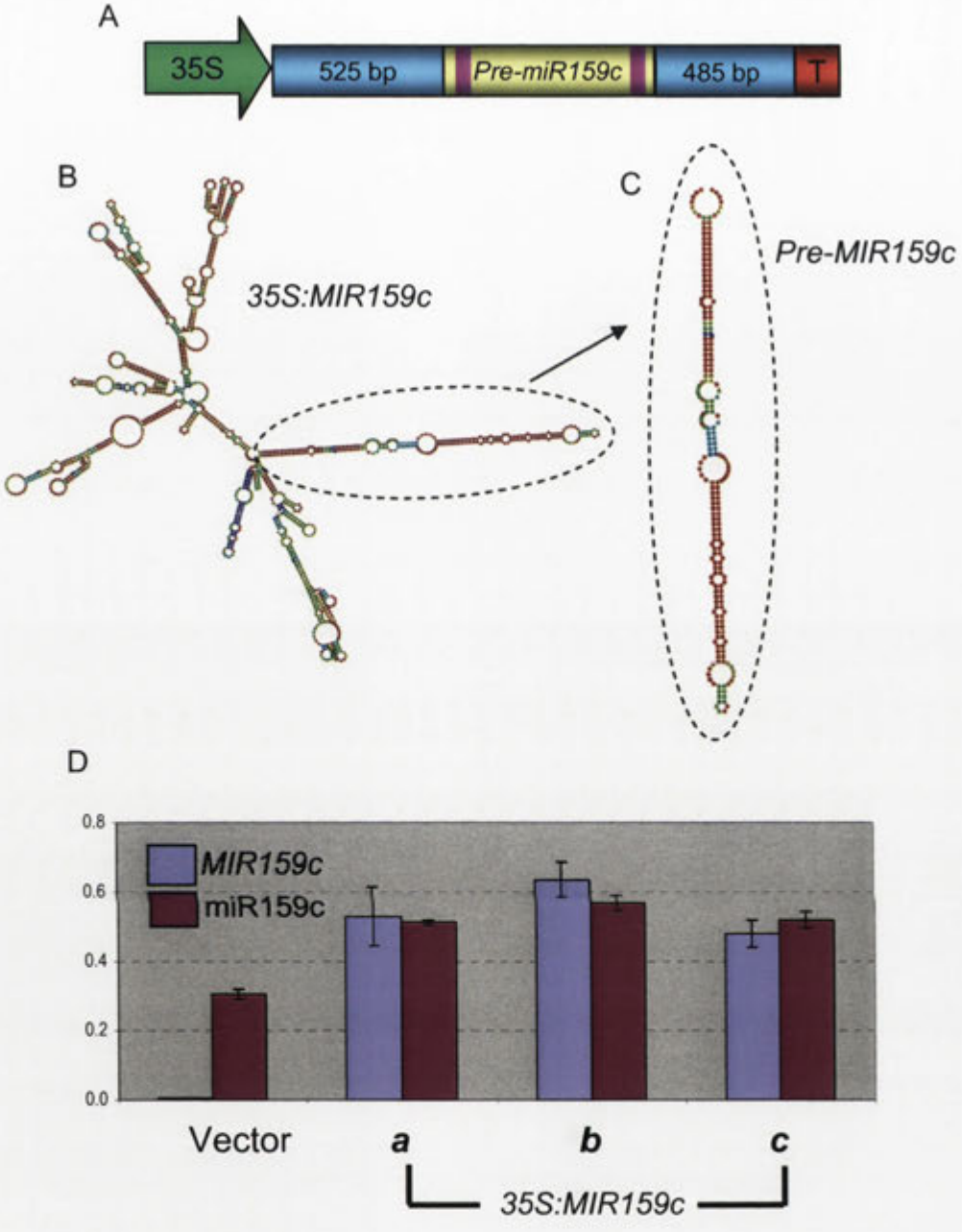


Fig 4: Over-expression of miR159c

(A) The *35S:MIR159c* construct used to transform Arabidopsis, with relevant regions shown. The pink bars represent miR and miR* sequences of MIR159c. 35S= tandem 35S promoter T= terminator. Figure is not to scale. (B) Predicted RNA fold of the *35S:MIR159c* sequence. The sequence used for folding was taken from 525 bp upstream to 485 bp downstream of *MIR159c* stem loop. (C) RNA fold of native miR159c stem loop, showing the loop structure folds similarly to the transgenic stem loop.

(D) Inflorescence tissue from three primary transgenic lines (a,b,c) and vector control was assayed by qRT-PCR to detect *MIR159c* transcript (blue panels) or qSL-PCR for miR159c (purple panels). At least six biological replicates were used for each RNA sample. Values represent mRNA levels relative to *Cyclophillin* for *MIR159c,* and miRNA levels relative to *sno101* for miR159c. Error bars represent SEM.

When miR159a is over-expressed, anther defects occur, attributed to downregulation of at *MYB33* (Achard et al., 2004; Schwab et al., 2005), and likely due to downregulation of at least also *MYB65*, as both these genes need to be disrupted to produce anther defects (Millar and Gubler, 2005). Because miR159c is highly similar to miR159a, and is bioinformatically predicted to downregulate *MYB33* and *MYB65,* it is possible that overexpression of miR159c could similarly result in anther defects. Twenty primary transformant plants that grew on selection for the selectable marker gene were planted to flowering, but all developed anthers and produced pollen indistinguishable from wild-type and empty vector control plants. PCR genotyping confirmed all plants tested were positive for the transgene. However the phenotypes of these plants appeared indistinguishable from wild-type (data not shown). This result accorded with the previously described report (Palatnik et al., 2007), that miR159c cannot be over-expressed to the extent that anther defects are produced as for miR159a overexpression.

6.2.4 miR159c is processed with very low-efficiency.

RNA was extracted from the inflorescences of *35S:MIR159c* plants and *MIR159c* transcript was measured using primers that annealed at the 3' end of the stem-loop (forward) and downstream of the stem-loop (reverse). In three independent transgenic plants the average level of *MIR159c* transcript increase was nearly 100-fold (Fig 6.7 D). Therefore the 35S promoter was able to over-express at least *MIR159c*. On the same RNA samples, measurement of mature miR159c was also carried out by qSL-PCR. The assays did show an increase in miR159c, but only 1.6 fold on average (Fig 6.7 D). Although cross-reaction of the miR159c assays RT-primer to miR159a and miR159b

would likely understate the absolute fold-changes, it is clear that the *35S:MIR159c* construct is unable to produce equivalent levels of mature miR159c.

This low level of miR159c is apparent when comparing the level of miR159c measured in transgenic plants compared to endogenous levels of miR159a and miR159b; although miR159c is likely artificially high due to cross-reaction with miR159a and miR159b, at an average relative expression level of 0.55, it is still considerably lower than miR159a (4.3) and miR159b (1.5) (Chapter 3). Thus even when over-expressed, miR159c is considerably lower than native levels of miR159a or miR159b. In summary, despite strong overexpression of *MIR159c*, low processing efficiency of mature miR159c prevents its high accumulation.

6.3 Discussion

6.3.1 Analysis of *MIR159c* suggests prior neo-functionalisation and subsequent obsolescence.

From deep sequencing studies of miRNAs across several species, it has been suggested that *MIRNA* genes undergo frequent birth and death (Axtell et al., 2007; Fahlgren et al., 2007; Rajagopalan et al., 2006). It is generally regarded that deeply conserved miRNAs tend to be highly expressed, while recently evolved miRNAs are initially lowly expressed. Subsequent fates of young miRNAs can range from death to stabilisation within regulatory networks (reviewed in Axtell 2008). However it is difficult to fit miR159c within this model. While belonging to an ancient family (albeit based only on its mature miRNA sequence), miR159c is lowly expressed, and was found to have no noticeable impact on *Arabidopis* development. Yet whether or not miR159c has ancient or recent origins, there is strong evidence that *MIR159c* previously carried out a specialised function that may have become obsolete.

6.3.2 Overlapping *MIR159c* and *GAMYB-like* transcription in the tapetum.

In contrast to *MIR159a* and *MIR159b*, *MIR159c* appears to be transcribed in the same discrete cell layer (the tapteum) as *MYB101* and *MYB33* (and possibly other *GAMYB-like* genes). Given this, it would be reasonable to imagine that co-transcription of *MIR159c* with other *GAMYB-like* genes may lead to their downregulation. This would be analogous to findings from chapters 3/4, where miR159a and miR159b were shown to regulate targets in co-transcribed domains (Allen et al., 2007). Furthermore in rice,

based on miR159 expression that correlated with *GAMYB* downregulation, it was claimed that miR159 regulates *GAMYB* in anthers (Tsuji et al., 2006). Therefore there is a suggestion from rice that miR159 can regulate anther *GAMYB* genes. *MIR159c*, being expressed in the same anther cells as *GAMYB-like* genes, would appear a likely candidate for this role in *Arabidopsis*.

6.3.3 *Arabidopsis* miR159c does not appear to regulate *GAMYB-like* genes in anthers

However in this chapter it was found that low transcription of *MIR159c*, coupled with low efficiency of miR159c processing, means this gene is unable to regulate potential targets. This was demonstrated primarily by the lack of any observable consequence in the *mir159c* knockout mutant. In particular anthers and pollen (where *MIR159c* appears to be transcribed) were examined by SEM, but appeared morphologically indistinguishable from wild-type. Analysis of the steady state mRNA levels of potential miR159c targets provided a molecular explanation for this, where there were no major mRNA changes to any of these targets.

6.3.4 Genomic context of *MIR159c* and inefficient processing may account for low expression of miR159c.

The presence of a transposable element 214 bp upstream of the *MIR159c* stem-loop may account for the low expression level of *MIR159c*. This element is also present in several closely related accessions of *Arabidopsis* (data not shown), therefore attenuation of *MIR159c* by this element may be a widespread and conserved process in *Arabidopsis*. This study has found that in addition to low transcription of *MIR159c*, additional processing inefficiencies further reduce miR159c accumulation (see also

below). However it is not clear what features of the *MIR159c* stem-loop render it unable to produce substantial levels of miR159c. Notably, pre-miR159c is predicted to contain several bulges larger than both *MIR159a* and *MIR159b* stem-loops in its middle-section (see Chapter 1, Fig 1.1). Although recently Bolgana et al (2009) demonstrated that the size of terminal stem-loop bulges can influence miRNA processing ability, it remains unclear to what extent internal bulges distinct from the mature miR/miR* sequence may influence accumulation of mature miRNAs. It would be interesting to determine if reduction of the *MIR159c* internal stem-loop bulges to equivalent sizes as in miR159a/b stem-loops may enhance miR159c processing. Overall this study has shown that miR159c does not appear to be processed as readily as miR159a and miR159b can be. Although not much attention has been paid to this area, processing of mature miRNA sequences from stem-loops may be a very important aspect in the regulation of miRNA expression.

6.3.5 Very low processing efficiency argues against an inducible role for miR159c.

Although under standard conditions, miR159c appears to be non-functional, the possibility that miR159c may play a role in different environmental circumstances was worthy of consideration- particularly as this has been shown for other miRNAs that have low expression under standard conditions (Ding et al., 2009; Jian et al., 2009). However by over-expressing *MIR159c*, this chapter demonstrated that even if *MIR159c* is able to be upregulated, processing of miR159c is still unable to produce sufficient miR159c to result in any phenotype in anthers and pollen indistinguishable from wild-type. This was demonstrated when even 100-fold changes in *MIR159c* transcript were insufficient to produce levels of miR159c that could render plants male sterile. This contrasted with miR159a overexpression (Achard et al., 2004; Schwab et al., 2005),

which led to male sterility attributed to downregulation of *MYB33* and *MYB65* (Achard et al., 2004). Because only a small degree of mature miR159 expression greater than wild-type was detected in *35S:MIR159c* plants, this may provide an explanation for why previous experiments using northern blotting failed to detect increased expression of miR159 in *35S:MIR159c* plants; small amounts of transgenic miR159c may not be detectable amongst the more highly abundant miR159a/miR159b background present in infloresence tissue outside anthers. It is also possible that the sequence of mature miR159c (that contains an additional *MYB33/MYB65* mismatch compared to miR159a) may account for the lack of male sterility observed; however this appears unlikely because the *35S:MIR159c* construct could compliment the *mir159ab* mutant (data not shown) suggesting this sequence, even when minimally overexpressed, can downregulate *MYB33* and *MYB65* to a certain extent.

6.3.6 miR159c regulation of *GAMYB* may represent an ancient regulatory module.

The fact that *MIR159c* appears to be co-transcribed with at *MYB101, MYB33* (and possibly other *GAMYB-like* genes) in the tapetum suggests that miR159c may have previously regulated these genes. However in *Arabidopsis* this requirement no longer appears important for two main reasons. Firstly, from the perspective of *MIR159c*, loss of this gene appears to have no noticeable molecular or phenotypical consequence. Secondly, from the perspective of potential miR159c targets, as has been demonstrated with *MYB101*, expression of this gene is largely independent of miR159 regulation (chapter 5). Furthermore, even if *MYB101* is overexpressed in anthers, there is no obvious detrimental phenotype, suggesting that although upward fluctuation in *GAMYB-like* levels can be tolerated, *GAMYB-like* downregulation in anthers is detrimental, as shown by overexpression of miR159a (Achard et al., 2004; Schwab et

al., 2005) or T-DNA knockout of *MYB33* and *MYB65* (Millar and Gubler, 2005). This notion is consistent with the inability of miR159c to noticeably downregulate *GAMYB-like* genes in anthers.

Despite the observation that miR159c appears not to regulate co-transcribed *GAMYB-like* genes in the tapetum, an insinuation is miR159c previously had regulatory importance. Evidence of this is provided by the phenotypes of *GAMYB* overexpresing transgenic barley (Murray et al., 2003), where such plants were found to produce anther defects. Although this implies endogenous barley miR159 is unable to suffciently downregulate overexpressed *GAMYB*, it also suggests that miR159 attenuation of *GAMYB* levels may be important in Barley, and possibly was for progenitor species of *Arabidopsis*. Thus it is possible that an earlier miR159c may have provided a "tuning" function in dampening *GAMYB* genes in anthers, although there is no evidence to suggest this is a current function in *Arabidopsis*. Rather, there is strong evidence that miR159c no longer has any function in *Arabidopsis*, either under standard conditions, or such conditions that may lead to upregulation of *MIR159c* transcript. Therefore regulation of anther *GAMYB-like* genes by miR159c likely represents a former, but now obsolete regulatory module in *Arabidopsis*.

Chapter 7

General Discussion

Nor ought we to marvel if all the contrivances in nature be not, as far as we can judge, absolutely perfect; and if some of them be abhorrent to our ideas of fitness… The wonder indeed is, on the theory of natural selection, that more cases of the want of absolute perfection have not been observed.

-Charles Darwin

7.1 Determination of miRNA importance in gene expression and development

The identification of miRNAs has led to questions regarding their function and relevance. Determining how miRNAs influence gene regulation and their relative importance in controlling development are current challenges. In plants, the importance of particular miRNAs in gene regulation has been largely inferred using three approaches; rendering targets miRNA resistant, recovery of miRNA guided cleavage products, and miRNA overexpression. The molecular and phenotypical consequences of these experiments have often implicated certain miRNA:target relationships to be relevant. However there remain very few examples of loss-of-function approaches taken in determining the roles particular plant miRNAs play in gene regulation.

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This study has employed all these approaches in investigating miR159, and all have been informative in revealing aspects of miR159 function and specificity. However the most salient findings have emerged from detailed characterisation of miR159 knockout mutants. This analysis has not only revealed a very discrete functional specificity of this miRNA family, but exposes possible limitations to the more commonly used methods in ascribing significance to miRNA:target relationships.

7.2 Functional redundancy of miR159a and miR159b

One of the proposed bottlenecks to finding loss-of-function mutants with observable phenotypes has been functional redundancy within *MIRNA* families (Sieber et al., 2007). This study substantiated this notion, demonstrating functional redundancy occurs in *MIRNA* families (Allen et al 2007), and provides an explanation for why *mir159* mutants have not been previously identified through forward genetic screens; both alleles of *MIR159a/MIR159b* need to be knocked out to produce any phenotype

distinguishable from wild-type. This is an encouraging finding, as further *MIRNA* families may be similarly characterised if knockouts can be produced for potentially redundant *MIRNA* genes. A further corollary of this result is miR159 is produced in substantial excess, as only one copy is sufficient to carry out miR159a/mir159b function. This fact, combined with the finding that *MYB33:GUS* is completely silenced in all tissues where miR159a/miR159b is expressed, implies that miR159 has strong silencing ability and could be described as a "switch" miRNA. This role is consistent with the developmental consequence of *MYB33/65* deregulation, where it was found deregulation of these genes is detrimental to growth. Additionally, the fact that miR159 is in substantial excess implies miR159 would need to be strongly downregulated to enable deregulation of *MYB33/MYB65*, and the implications of this will be discussed later.

7.3 The functional specificity of miR159

The finding that all pleiotropic phenotypes of *mir159ab* could be eliminated by removing *MYB33* and *MYB65* explicitly demonstrated the discrete functional specificity of miR159a/miR159b. This was largely explained by differences in expression domains, where *MYB33* and *MYB65* appeared to be the only predicted targets transcribed in the domain of *MIR159a/MIR159b*. Other targets (*MYB81,97,101,104,120,DUO1*) appeared to be transcribed in anthers and pollen, and although miR159 guided cleavage products for some of these could be isolated, miR159 regulation of these genes is sufficiently minimal that it appears to have no biological relevance. This claim is supported by the wild-type phenotype of the *mir159ab/myb33/myb65* mutant, and by the lack of deregulation for these genes in *mir159c*, despite being transcribed in the same domain. Additional evidence of this discrete specificity was supported by investigating the

relevance of miR159 regulation for *MYB101*, the most highly expressed *GAMYB-like* gene in the inflorescence. Here it was found that expression of this gene appeared largely independent of miR159 regulation. In contrast, *MYB33* is efficiently silenced in all tissues except in anthers where miR159 appears not to be expressed. These contrasting results suggest the repression of *MYB33/MYB65* by miR159a/b has evolved to be highly efficient, whereas this is not the case for *MYB101*; ectopic transgenic expression of this gene was not silenced as efficiently by miR159, even when it contained its native miR159 site, in contrast to previous *MYB33* transgenic experiments (Millar and Gubler., 2005, Palatnik., 2003).

7.4 Implications from the discrete specificity of miR159.

From the finding that miR159 has a such discrete functional specificity, there are several implications for the assessment of other potential miRNA:target relationships. Firstly, it has recently been shown that for miR398, regulation of two low-complemtarity targets by translational repression can occur (Dugas and Bartel, 2008), and it has been further shown that translational repression is widespread in plants (Brodersen et al., 2008). From these findings it has been postulated that translational repression of low complemetarity targets in plants may be widespread (Brodersen and Voinnet, 2009), as in metazoans. However the results from this study argue that if this is the case, then at least for any potential low complementarity miR159 targets, such regulation would be of little or no importance. This is demonstrated by the quadruple *mir159ab/myb33/myb65* mutant, where the absence of additional phenotypes demonstrates any possible deregulation of putative low complementarity targets has no discernable biological impact.

A second implication is related to the usefulness of using bioinformatics, miRNA overexpression, or 5'-RACE to insinuate miRNA:target relationships. Using several different bioinformatics programs, this study predicted at least twenty genes may be miR159 regulated. Additionally, other studies have isolated miR159-guided cleavage products, and have observed downregulation of several of these predicted targets by overexpression of miR159 (Schwab et al., 2005). This study has also recovered cleavage products for *MYB101* and *MYB81*. Yet the loss-of-function approach taken here demonstrates that predictions and transgenically induced target down regulation by miRNA over expression does not always imply a miRNA:target relationship has biological relevance.

By example, Schwab et al (2005) found that *MYB33* and *MYB65* were not transcriptionaly downregulated by overexpression of miR159a, but rather *MYB101* was the most strongly downregulated gene. However the fact that anthers were male sterile, similar to *myb33/myb65* mutants, suggests that the downregulation of *MYB101* could be a secondary effect, possibly due to *MYB33/MYB65* translational repression in anthers; this is substantiated by the observation that *MYB101* levels are considerably lower in *myb33/myb65* anthers (Tony Millar, personal communication). Given that miR159a overexpression produces only anther defects, in contrast to widespread pleiotopic phenotypes that result from loss of miR159a/b function, inferences from overexpression alone would seem at least insufficient, and possibly misleading in describing miRNA:target relationships. This study demonstrates that to investigate the significance of miRNA regulation, a range of approaches need to be taken, and using knockout mutants of miRNA genes is the most revealing method in determining *in-vivo* importance of miRNA:target relationships.

7.5 miR159c regulation of anther/pollen transcribed genes may represent an obsolete module.

The fact that the major targets of *invivo* relevance for miR159a and miR159b were found to be *MYB33* and *MYB65* led to an obvious question: what is the significance of miR159 target sites in other genes not apparently regulated by miR159a or miR159b? This question was approached from several angles. Firstly, it was sought to determine if other genes were subject to miR159/miR319 cleavage based regulation by using sensitive nested 5'-RACE. Secondly, using *MYB101* as a representative example of a non-deregulated target in *miR159ab*, it was sought to determine if miR159 regulation may have any *in vivo* importance for these anther/pollen transcribed *MYB* genes, where miR159a and miR159b appear to be absent. Finally, the possibility that regulation by miR159c may account for the presence of miR159 sites in other targets was investigated primarily by functional analysis of the *mir159c* knockout mutant. The result of these approaches found that miR159c does not significantly regulate these genes, and at least for *MYB101*, the miR159 target site appears largely inconsequential for its expression. Combined with the fact *MIR159c* appears to be transcribed in the same domain as *MYB101* and other miR159 targets, this suggested that miR159c may have been responsible for regulation of these genes previously, but in *Arabidopsis* such regulation is no longer a requirement for normal development.

The notion of this non-functional miRNA regulatory system could be challenged by reasoning that it may actually serve to confer robustness to expression of these other genes, when facing different and/or stressful environmental conditions. A similar

concept has been motivated from observations in nematodes and insects, where, like for miR159c, there are often no developmental phenotypes associated with loss of conserved miRNAs (Li and Carthew, 2005; Miska et al., 2007), suggesting selection has maintained some miRNA targets relationships for non-obvious roles. Recent experimental evidence has validated this, where *Drosophila* miR-7 was found to buffer miRNA regulatory networks against environmental fluctuations when exposed to temperature changes (Li et al., 2009). Likewise, the importance of the mice miR-223 was made apparent only after subjecting mice carrying mutations in miR-223 miRNA to immune challenge (Johnnidis et al., 2008). Similarly, the possibility that miR159c regulation of *MYB101* and other targets may be latent, until required under different environmental conditions, could conceivably provide an explanation for its apparent non-functionality under normal growth conditions.

However, although this possibility cannot be dismissed, evidence from this study would argue against this, and rather supports the notion that miR159c:target regulation represents a non-functional module, regardless of environmental circumstances. There are several lines of evidence supporting this. Firstly, overexpression of *MIR159c* was unable to result in major changes to mature miR159c levels. This suggested that even if *MIR159c* were able to be induced under different environmental conditions, intrinsic low processing efficiency would prevent this miRNA from impacting on anther development. This was evident in the wild-type appearance of anthers and pollen in *35S:MIR159c* transgenic plants.

In regards to possible *MYB* target genes, when *MYB101* was overexpressed in inflorescences, it produced no phenotype additional to wild-type, indicating if any environmental conditions were to lead to up-regulation of this gene, there is no

observable consequence to higher *MYB101* expression. Concomitantly there would be no strong selective pressure maintaining a backup system to ensure *MYB101* was downregulated, even if normal transcriptional and posttranscriptional controls were inadequate. Only when ectopically expressed in rosettes, transgenic *MYB101* was shown to produce phenotypes, but this was shown to be at least partially independent of miR159 regulation, as such phenotypes occurred in both wild-type *MYB101* and miR159 resistant *mMYB101* transgenic plants.

7.6 Specialisation and loss-of-function within *MIRNA* families

It is interesting that analysis of an entire miRNA family has revealed a range of evolutionary fates for different members. miR159a and miR159b appear to be ingrained and central to *MYB33* and *MYB65* gene regulation, while miR159c appears to be non-functional, or on an evolutionary path to becoming as such. Similarly, analysis of the closely related miR319 family in Brassicaceae has revealed functionality can be lost for specific members (Warthmann et al., 2008), thus it appears even duplicated conserved miRNAs can specialise or become obsolete. Interestingly, a recent study has suggested various *Arabidopsis* accessions process different levels of miR824 based on variations within their *MIR824* stem-loop structures (de Meaux et al., 2008). This would appear to be at least partially the case for miR159c, where although mature miR159 sequences are virtually identical, differences between miR159c and miR159a/b stem-loops may account for differences in their abundance. Overall, it has been shown that substantial functional differences can exist within a single miRNA family. This is not surprising considering gene duplication and subsequent neo-functionalisation are common facets of evolution.

7.7 Further unknown determinants besides target sequence influence target down-regulation

In plants, bioinformatic predictions and analysis of miRNA:target relationships has tended to focus on the 20-24 nt region of complementarity between the mature miRNA sequence and the mRNA target site. This study has shown such parameters are insufficient to adequately describe or predict whether a miRNA is capable of sufficiently downregulating a target. This was demonstrated where both *MYB101* genomic and *MYB101:GUS* transgenes could result in phenotypes attributed to increased *MYB101* transcript, indicating transgenic *MYB101* was unable to be downregulated by miR159 to the same extent as *MYB33*. This reflects a similar report by Palatnik et al (2007), where *DUO1*, another miR159 target, when ectopically expressed with its native miR159 site, could also lead to aberrant phenotypes. These results contrast with *MYB33*, where no phenotypes different to wild-type have been reported when this gene is overexpressed (Millar and Gubler, 2005; Palatnik et al., 2003). This finding shows the picture of miRNA:target downregulation is more complex than miRNA:target site region complementarity, and suggests that other factors may influence target downregulation. Given this is a little explored area in plant miRNA function, it would be interesting to determine if other factors identified in animal miRNAs systems, such as target site accessibility (Kertesz et al., 2007; Long et al., 2007) and RNA binding proteins (Kedde et al., 2007), may also contribute to plant miRNA:target silencing efficiency.

7.8 A model for miR159 regulation of target genes

From the results presented here and throughout this thesis a proposed model describing the relationship between miR159 and potential targets is shown in Fig 7.1. To summarise: miR159a/miR159b predominantly regulates *MYB33/MYB65* except in anthers (dashed black box). This has been demonstrated by overlap of transcriptional domains (Chapter 3), the widespread deregulation of *MYB33* and *MYB65* in *mir159ab*, and by the wild-type vegetative appearance of the *mir159ab/myb33/myb65* mutant (Chapter 4), demonstrating the discrete specificity of this regulatory module. The prediction of Achard et al., (2004) that *MYB33* or *MYB65* might operate in a feedback loop to upregulate *MIR159a/b* appears unlikely, as *MIR159a* and *MIR159b* transcripts were unaffected in *mMYB33* lines (Chapter 4). Transcripts of *MYB81*, *MYB101* and other genes are shown in the domain of miR159a/miR159b, based on the isolation of their cleavage products in wild-type in this study, or in other studies (Alves-Junior et al., 2009; German et al., 2008), in contrast to their relative scarcity in *mir159ab* (Chapter 5). For *DUO1*, *MYB101* and possibly at least *MYB81*, this may represent spurious transcription outside their normal domain (indicated by arrow). The scarcity of these cleavage products, and the fact that *mir159ab/myb33/myb65* has a wild-type appearance except in anthers, indicates that although *MYB81/MYB101* and other transcripts can be cleaved by miR159a/b, this has no discernable impact on development. This is represented in the model by their smaller type.

miR159c is shown in anther/pollen cells with *GAMYB-like* genes. This is based on GUS reporter expression patterns (Chapters 5 and 6, Millar and Gubler., 2005) showing *MIR159c* and *MYB33/MYB101* share transcriptional domains, and Affymetrix data for some of the other *GAMYB-like* genes (Chapter 4). The dotted red downregulation symbol indicates miR159c may have previously regulated these genes (based on expression domains and miR159 sites in these targets). The cross indicating this does not occur reflects the fact *mir159c* has a phenotype indistinguishable from wild-type (Chapter 6), and also *mir159abc* mutants are indistinguishable from *mir159ab* (Chapter 3). This is also supported by the finding that no targets are transcriptionally deregulated in *miR159c*, and overexpression of *MIR159c* is unable to produce male sterility (Chapter 6).

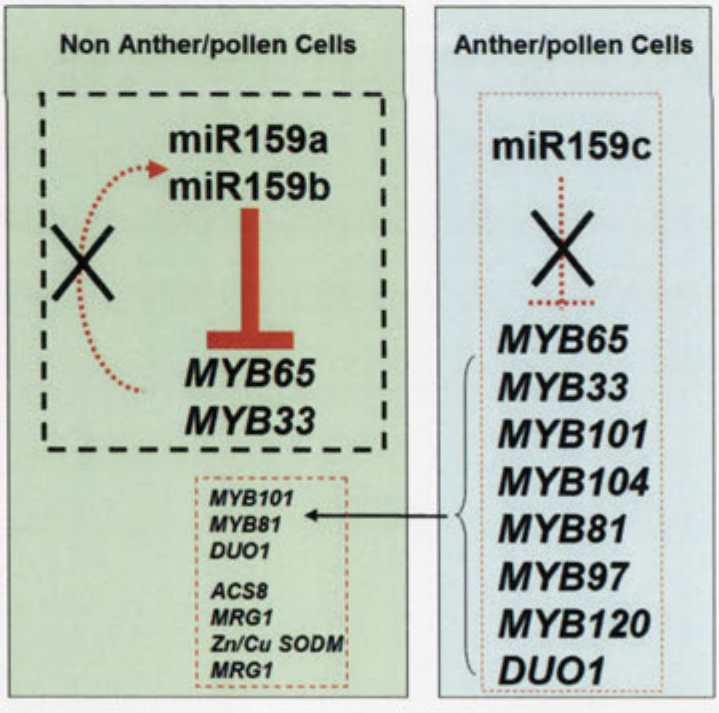

Fig 7.1: Model of miR159 regulation of targets in *Arabidopsis*
See text for explanation

7.9 Possible functions of the miR159a/miR159b:*MYB33/MYB65* regulatory module.

A final conundrum is the relevance of the miR159a/miR159b:*MYB33/MYB65* regulatory module. The wild-type appearance of the *mir159ab/myb33/myb65* mutant (except in anthers, which have the *myb33/myb65* male sterile phenotype) demonstrates that this module is dispensable under standard growth conditions. This presents an obvious question: Why has this module been selected? Along a similar vein, why would *Arabidopsis* expend energy widely transcribing four sets of genes, only to have two expressed in a very discrete domain? It is tempting to speculate that under different conditions, downregulation of miR159 would allow deregulation of *MYB33/MYB65*. However this is hard to reconcile with two facts. Firstly, deregulation of *MYB33* and *MYB65* as demonstrated in *mir159ab*, appears deleterious. Secondly, deregulation of *MYB33* and *MYB65* would require substantial downregulation of miR159, as these miRNAs have been shown to be produced in considerable excess.

Yet it is possible that aspects of the *mir159ab* phenotype may actually be beneficial under different environmental conditions. The *mir159ab* mutant has upwardly curled leaves and is stunted compared to wild-type, but many other plants share these characteristics that help them survive in particular environments (Heckathorn and DeLucia, 1991). Additionally, the fact that *MIR159a* and *MIR159b* appear to not be transcribed in anthers suggests that under certain circumstances, transcription of these genes can be downregulated to the extent that MYB33 and MYB65 protein is expressed. Therefore it is possible that down-regulation of miR159, allowing up regulation of *MYB33* and *MYB65* may be a regulatory function that has been selected for.

Yet at the opposite extreme of possibilities, the regulatory module may simply be a result of evolutionary convenience, but not necessarily genetic efficiency. As *MYB33* and *MYB65* are required for pollen development, it may be that these genes evolved so they are transcribed broadly, and to maintain anther specific expression in the inflorescences miR159 regulation is required. Nature is replete with comparable situations; a highly similar system has been shown to operate in plant sperm cell development, where expression of the *LGC1* gene is only permitted in sperm cells, because it is otherwise repressed by germline-restrictive silencing factor (GRSF) which is transcribed ubiquitously except in sperm cells (Haerizadeh et al., 2006). Comparable systems also operate in vertebrates to specify neuron gene expression (Lunyak and Rosenfeld, 2005). These virtually analogous systems all present essentially the same question asked earlier: why not have a gene specifically under transcriptional control, rather than have it widely transcribed and elaborately regulated to achieve essentially the same effect- particularly when the regulators themselves are also subject to transcriptional controls?

This thesis cannot answer this broad question as it applies to the miR159a/b:*MYB33/MYB65* regulatory module. However if there is a further requirement for this module, experiments that subject the *mir159ab/myb33/myb65* mutant to different environmental stresses may help determine this. Additionally, studies focused on downstream targets of *MYB33* and *MYB65* may reveal the biological process these genes control in *Arabidopsis*. These experiments may shed further light on how miRNAs affect gene expression and control development.

CHAPTER 8
References

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Chapter 9 Appendix

Primer table I

Name	Template	sequence 5-3'	purpose
fwd:MYB33(2)	At5g06100	TCGTCATCTCCTCCACACTCTG	qRT-PCR total *MYB33*
rv:MYB33(2)	At5g06100	CCTCGGATTTAGTTTGGGATAC	qRT-PCR total/uncleaved *MYB33*
fwd:MYB33(3)	At5g06100	CCAGATAGCCATACCCCTACG	qRT-PCR of uncleaved *MYB33*
MYB33_endo159site	At5g06100	CTGAATATTGGAATGAAGGGAGC	qRT-PCR specific for endogenous *MYB33*
fwd:MYB65(1)	At3g11440	CTTCCCCAAAGCAAATCTG	qRT-PCR of total/uncleaved *MYB65*
rev:MYB65(1)	At3g11440	TTCACTGCCCCAAACAAG	qRT-PCR of total/uncleaved *MYB65*
fwd:MYB101 uc	At2g32460	CGAGTTCTTTCCCTTTAGGACT	qRT-PCR of uncleaved *MYB101*
rv:MYB101(1)	At2g32460	TGGCTCATTGTACTTGTTGTG	qRT-PCR of uncleaved *MYB101*
MYB101_1069F	At2g32460	GTCCATCTTGAGCCACCTTCTG	qRT-PCR of total *MYB101*
MYB101_1308R	At2g32460	TGGCTCATTGTACTTGTTGTG	qRT-PCR of total *MYB101*
MYB81 1026F	At2g26960	AACACTTTGGTTCAATCTCCTCTG	qRT-PCR of total *MYB81*
MYB81 1179R	At2g26960	ATGACTGAAACAGTGAAGATTCTG	qRT-PCR of total *MYB81*
MYB97 919F	At4g26930	GGTTTGCATACAAATACCTGTCAG	qRT-PCR of total *MYB97*
MYB97 1050R	At4g26930	GTTGTTGTTGTTGTTGTTGTCCTC	qRT-PCR of total *MYB97*
MYB104 907F	At2g26950	GCAGAACAATATAACCCAATGCTG	qRT-PCR of total *MYB104*
MYB104 1000R	At2g26950	ATGATGATGGGAAATCTGTTGGTG	qRT-PCR of total *MYB104*
MYB120 1300F	At5g55020	AACTTCACAGACAACGAGAGACAG	qRT-PCR of total MYB120
MYB120 1468R	At5g55020	TCGGAAGAGAAGCTGTGAGTTGTC	qRT-PCR of total MYB120
TCP4 1325F	At3g15030	TGGTTTGATCCTCACCATCATCAC	qRT-PCR of total *TCP4*
TCP4 1428R	At3g15030	GATTCCGGGGATTGCTGATTGGTG	qRT-PCR of total *TCP4*
TCP2 2180F	At4g18390	GTCATTCCTCGCTAATCTACAGAG	qRT-PCR of total *TCP2*
TCP2 2285R	At4g18390	CTGGTGATTGTGGTGGTGATTCTC	qRT-PCR of total *TCP2*
cyclophilin F	At2g29960	TGGACCAGGTGTACTTTCAATGG	qRT-PCR reference gene
cyclophilin R	At2g29960	CCACTGTCTGCAATTACGACTTTG	qRT-PCR reference gene
MYB125 300F	At3g60460	TGTGAAGAATTTCTGGAGTAGCAG	qRT-PCR *MYB125* total
MYB125 447R	At3g60460	AGAGGATTGACGGATTGGTTTGAC	qRT-PCR *MYB125* total
rv:miR159a(1)	At1g73687	CACGCTAAACATTGCTTCGGA	qRT-PCR *MIR159a/mir159a*
rv:miR159a(2)	At1g73687	TCTCATCTACCCGAGGCAGT	qRT-PCR *MIR159a/mir159a*
rv:miR159b(1)	At1g18075	TACATAACTGAAAAGTACGAAACTAATGG	qRT-PCR *MIR159b/mir159b*
rv:miR159b(2)	At1g18075	CAAAGTACAAACCATAAAAATTGC	qRT-PCR *MIR159b/mir159b*
rv:miR159c(1)	At2g46255	GCATCAACCACAAACACCTG	qRT-PCR *MIR159c/mir159c*
rv:miR159c(2)	At2g46255	CGTCTTCTCGTAAATAAACAACATT	qRT-PCR *MIR159c/mir159c*
a2S_159aF3	At1g73687	CGATAGATCTTGATCTGACGATGG	qRT-PCR *MIR159a* (upstream of *mir159a-2*)
a2S_159aR2	At1g73687	TCAATCCAAAGAAGAGTAAAAGCC	qRT-PCR *MIR159a* (upstream of *mir159a-2*)
JL202	pAC161	CATTTTATAATAACGCTGCGGACATCTAC	T-DNA genotyping
LB3	pCSA110/pDAP101/pAC161	TAGCATCTGAATTTCATAACCAATCTCGATACA	T-DNA genotyping *159a/b/c*
MYB33 2331F	At5g06100	GAAGAACAGCTTATCGTTGAATTGC	T-DNA genotyping *myb33*
MYB33 2548R	At5g06100	CGTTGTCGCCTCTTGATACGAGTG	T-DNA genotyping *myb33*
MYB65 1454F	At3g11440	TTACCTGGTCGAACAGATAATGAG	T-DNA genotyping *myb65*
MYB65 1625R	At3g11440	GATGTCTTCTTCTTCTATCTACTC	T-DNA genotyping *myb65*
RA_o2588	pAC161	CGCCAGGGTTTTCCCAGTCACGACG	T-DNA genotyping *mir159a-2*
RA_o8760	pAC161	GGGCTACACTGAATTGGTAGCTC	T-DNA genotyping *mir159a-2*
QRT1_F	AT5G55590	CTTCCTTTCTTTAACTCTCTCACTCCCAC	screening of *qrt1* in *mir159c*
QRT1_R	AT5G55590	TCATTATTGTTACCTATCTGCAACGCACAC	screening of *qrt1* in *mir159c*

Primer Table II

Name	Template	Sequence 5'-3'	Purpose
MYB33 1426R	At5g06100	CTCCTCGGATTTAGTTTGGGATACAGTAACCTG	5' RACE *MYB33* cleavage 1st round
MYB33 1354R	At5g06100	GGCTTCCAGAAGCAACATATCGAGCAGACCAG	5' RACE *MYB33* cleavage nested round
MYB101 1425R	At2g32460	GTTTTGCATTTCTGTCACCCGATACCAATCAG	5' RACE *MYB101* cleavage 1st round
MYB101 1166R	At2g32460	CTAACGTCCTTGAAGAGTCCGCCTCGAGAC	5' RACE *MYB101* cleavage nested round
MYB81 1169R	At2g26960	CAGTGAAGATTCTGAATCGGTATCGGTTGC	5' RACE *MYB81* cleavage 1st round
MYB81 1072 R	At2g26960	CAATCTGATGGTGTTTGAGCTGTCAGAGGAG	5' RACE *MYB81* cleavage nested round
MYB97 1315R	At4g26930	GTTGTTATCACAGAGGATTGCCCGTTTGAG	5' RACE *MYB97* cleavage 1st round
MYB97 1035R	At4g26930	GTTGTCCTCAAGCGCAGCCATGATTTGTCTC	5' RACE *MYB97* cleavage nested round
MYB97_1688R	At4g26930	CTGTTGGAAGAAACACGATGTCCGAAACCTC	5' RACE of *MYB97* cleavage 1st round
MYB97_1600R	At4g26930	GCTTAGGAGCACGAAAGCTATCAGCTAGAG	5' RACE *MYB97* cleavage nested round
MYB104_1002R	At2g26950	GAATGATGATGGGAAATCTGTTGGTGTCTG	5' RACE of *MYB104* cleavage 1st round
MYB104_1077R	At2g26950	GGTATCGGTTTTCGGTATTTCTCCTAATGAACC	5' RACE *MYB104* cleavage nested round
pGEM_2488_F	pGEM-T/Teasy	GCCAGTGAATTGTAATACGACTC	screening/sequencing pGEM-T/pGEMT easy
pGEM_2510_R	pGEM-T/Teasy	CTCAAGCTATGCATCCAACG	screening/sequencing pGEM-T/pGEMT easy
MYB101G_GW_F	At2g32460	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTCGTGTCTGGCTATGCCCGGAGAG	*MYB101* genomic/GUS gateway construct
MYB101G_GW_R	At2g32460	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAATGTAATTTCTGACATTTTCAGG	*MYB101* genomic gateway construct
MYB101Tr_GW_R	At2g32460	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGATGGTGGTGGAGAGACGACG	*MYB101* GUS gateway construct
pDONORZ_471F	MYB101Genomic/GUS entry	TTGATGCCTGGCAGTTCCCTACTCTC	gateway construct screening
MYB101g_398F	MYB101Genomic/GUS entry	GATTCGACTTCAGTTGAACAGTTGTG	*MYB101* Genomic/GUS construct sequencing
MYB101g_995F	MYB101Genomic/GUS entry	CAGCAAGAACGTCTTCTACACAAGTACG	*MYB101* Genomic/GUS construct sequencing
MYB101g_1596F	MYB101Genomic/GUS entry	GTTTATAGGTTCTAGTGTTCTACTAATGTG	*MYB101* Genomic/GUS construct sequencing
MYB101g_2269F	MYB101Genomic/GUS entry	GAGCAAGAAGAAAGACATAAAAGGCGTGTG	*MYB101* Genomic/GUS construct sequencing
MYB101g_2854F	MYB101Genomic/GUS entry	GGTCTAAAGACAGACAATTATCTAAGTC	*MYB101* Genomic/GUS construct sequencing
MYB101g_3473F	MYB101Genomic/GUS entry	AAGATCATCATCGACCTTCACGCTAAGC	*MYB101* Genomic/GUS construct sequencing
MYB101g_4162F	MYB101Genomic/GUS entry	CTCTTTACAACAATAGCCTTGAGAATGAC	*MYB101* Genomic/GUS construct sequencing
MYB101g_4786F	MYB101Genomic/GUS entry	CTACTGGATCTTAGGGCTAATTGAC	*MYB101* Genomic/GUS construct sequencing
MYB101g_5401F	MYB101Genomic/GUS entry	CAAGTAAAAGAATTGCATTATACAAACTG	*MYB101* Genomic/GUS construct sequencing
MYB101g_6060F	MYB101Genomic/GUS entry	GGATATATTGATGTCACTCACTCTACAG	*MYB101* Genomic/GUS construct sequencing
mMYB101_F	MYB101Genomic/GUS entry	CAATAGCGTCCTCGAGCTTCCAAGCAATCAGCGACCGACCCATTCG	construction of m*MYB101*
mMYB101_R	MYB101Genomic/GUS entry	CGGTCGCTGATTGCTTGGAAGCTCGAGGACGCTATTGTCTAGTCCTAAAG	construction of m*MYB101*
Gate_RV2	MYB101Genomic/GUS	CTTTATGCTTCCGGCTCGTATGTTGTGTG	screening of *MYB101* transgenic plants
mMYB101scrF	MYB101Genomic/GUS	AGCGTCCTCGAGCTTCCAAGCAAT	screening of m*MYB101* transgenic plants
159c_GW_821F	At2g46255	GGGGACAAGTTTGTACAAAAAAGCAGGCTGATGGTCTTACGAATGCCTAGATTCTC	35S:*159c* gateway construct
159c_GW_2045R	At2g46255	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTATCGACTACAAGCAACCCAGCTG	35S:*159c* gateway construct
miR159a	miR159a	AAGAGCTCCCCTTCAATCCAAA	oligo probe for miR159 blot

Genetic analysis reveals functional redundancy and the major target genes of the *Arabidopsis* miR159 family

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Currently, there are very few loss-of-function mutations in microRNA genes. Here, we characterize two members of the *Arabidopsis MIR159* family, miR159a and miR159b, that are predicted to regulate the expression of a family of seven transcription factors that includes the two redundant *GAMYB-like* genes, *MYB33* and *MYB65*. Using transfer DNA (T-DNA) insertional mutants, we show that a *mir159ab* double mutant has pleiotropic morphological defects, including altered growth habit, curled leaves, small siliques, and small seeds. Neither *mir159a* nor *mir159b* single mutants displayed any of these traits, indicating functional redundancy. By using reporter-gene constructs, it appears that *MIR159a* and *MIR159b* are transcribed almost exclusively in the cells in which *MYB33* is repressed, as had been previously determined by comparison of *MYB33* and *mMYB33* (an miR159-resistant allele of *MYB33*) expression patterns. Consistent with these overlapping transcriptional domains, *MYB33* and *MYB65* expression levels were elevated throughout *mir159ab* plants. By contrast, the other five *GAMYB-like* family members are transcribed predominantly in tissues where miR159a and miR159b are absent, and consequently their expression levels are not markedly elevated in *mir159ab*. Additionally, *mMYB33* transgenic plants can phenocopy the *mir159ab* phenotype, suggesting that its phenotype is explained by deregulated expression of the redundant gene pair *MYB33* and *MYB65*. This prediction was confirmed; the pleiotropic developmental defects of *mir159ab* are suppressed through the combined mutations of *MYB33* and *MYB65*, demonstrating the narrow and specific target range of miR159a and miR159b.

development | functional specificity | micro-RNA | gene regulation

Micro-RNAs (miRNAs) are 20- to 24-nucleotide (nt) small RNAs that guide the RNA-induced silencing complex in a sequence-specific manner to target mRNA(s), regulating their expression either through degradation of the transcript or translational attenuation (1). They are derived from longer noncoding RNA precursors known as primary (pri) miRNAs, being processed from these transcripts by RNase III-like enzymes known as DICER-LIKE via multiple cleavage steps (2). Their requirement for development has been well characterized. In *Arabidopsis*, miRNAs have been shown to play critical roles in stem cell formation, organ identity, leaf polarity, vascular differentiation, and cell division patterns (3). Currently, there are ≈180 known miRNA loci in *Arabidopsis*, many of which are highly conserved across the plant kingdom (3–5). For instance, the miR159 family has been found in all examined seed-bearing plants (4). In *Arabidopsis*, this family is encoded by three genes, *MIR159a*, *MIR159b*, and *MIR159c*, located in different regions of the genome (6, 7). As determined by deep sequencing, miR159a and miR159b are highly expressed compared with miR159c (5). Their mature products are 21 nt long, with miR159a and miR159b only differing in sequence at one nucleotide, whereas miR159a and miR159c differ at two nucleotides (6). These sequence differences, together with unknown expression patterns of these individual miRNAs, mean that it is uncertain whether they target similar or distinct genes.

One known target of miR159 in *Arabidopsis* is *MYB33*, which belongs to a *GAMYB-like* family of transcription factors (8). In *Arabidopsis*, there are seven members in this family, all of which share a conserved putative miR159-binding site (9). Two of these genes, *MYB33* and *MYB65*, function redundantly. This is based on strong sequence similarity, expression patterns, and genetic analysis, where only a *myb33/myb65* double mutant displays phenotypic defects (9). *MYB33* has been the focus of miR159 regulation. The isolation of miRNA-guided cleavage products for *MYB33* (10, 11) and *in planta* assays (11) have demonstrated that miR159a cleaves *MYB33* mRNA. Mutation of the miR159-binding site (without changing the amino acid sequence of the gene) within *MYB33*, generating the mutant allele known as *mMYB33* (10), resulted in dramatic expansion of the expression pattern (9). For instance, expression of the *MYB33:GUS* reporter gene construct was only detected in anthers and in seeds, whereas the *mMYB33:GUS* reporter gene has strong expression in root and shoot apices and many floral organs in addition to anthers (anther filaments, carpels, sepals, and receptacles) (9). Furthermore, transgenic *mMYB33* plants have pleiotropic developmental defects, having curled/rounded leaves, stunted growth, and altered apical dominance. In contrast, transgenic *MYB33* plants have none of these developmental defects, indicating that miRNA control of *MYB33* regulation is absolutely critical for proper plant development (9, 10). This phenotype is dramatically different than a loss-of-function *myb33/myb65* mutant (9) or plants overexpressing miR159a precursors (*35S:miR159a*), where the only observable morphological phenotype is male sterility (11, 12).

Currently, only a small number of loss-of-function mutants in miRNA genes have been reported in any organism, which is counterintuitive to the notion that their influence is widespread and that they play pivotal roles in development (13). Genetic redundancy has been proposed as a possible contributing factor to this conundrum. Here, we show that this is the case for the *Arabidopsis* miR159 family, where only a *mir159ab* double mutant exhibits pleiotropic development defects. Based on the characterization of the spatial expression pattern of the *MIR159* genes, and combined with molecular and genetic analyses, we

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The authors declare no conflict of interest.

Abbreviations: miRNA, micro-RNA; T-DNA, transfer DNA; pri, primary.

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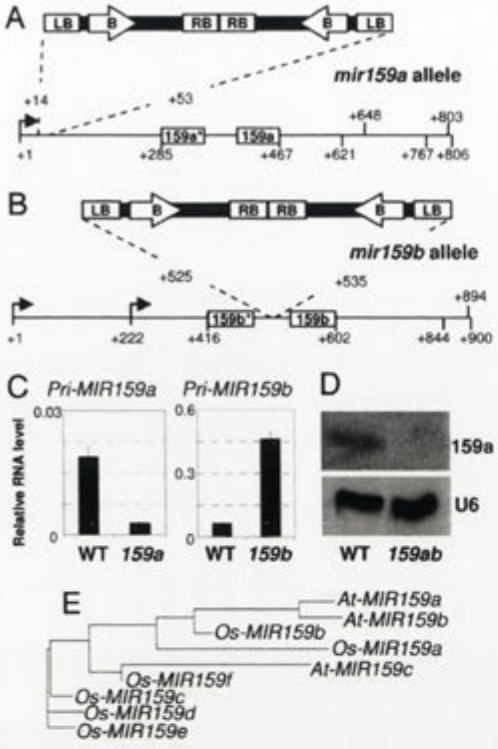

Fig. 1. Characterization and structure of the *MIR159* loci. Mapping of the pri-*MIR159* transcripts and the T-DNA insertion sites for *MIR159a* (*A*) and *MIR159b* (*B*). LB, left border; RB, right border; B, Basta-resistant gene. Arrows indicate transcriptional start sites with numbers indicating relative positions of the stem–loop regions and the varying polyadenylation sites. In both instances, the T-DNA loci were tandem inverted insertions because both plant–T-DNA junctions were isolated by using left border primers. (*C*) Relative RNA levels of pri-*MIR159* transcripts as determined by quantitative RT-PCR on RNA prepared from seedlings of wild-type, *mir159a*, and *mir159b* plants. (*D*) RNA gel blot analysis of mature miR159 levels in 72-h imbibed seeds. (*E*) A phylogenetic tree based on the stem–loop sequences of rice and *Arabidopsis MIR159* genes.

have found that the major targets of these miRNAs are even more limited in scope than previously had been predicted by using bioinformatics or overexpression strategies.

Results

Genomic Structure of the *MIR159* Genes. The primary transcripts of *MIR159a* and *MIR159b* were defined by using 5′- and 3′-end rapid amplification of cDNA ends (RACE) on RNA isolated from imbibed seeds. For *MIR159a*, a single transcriptional start site was mapped 446 bp upstream of the mature miR159a sequence, an identical position to where the transcription start-site of this gene had been previously mapped (14). In contrast, five different polyadenylation sites were found at the 3′ end from the analysis of an equal number of RACE clones (Fig. 1*A*), implying that the length of the 3′ end was highly variable. The largest transcript of *MIR159a* was 806 nt long. Similarly, variable polyadenylation sites were mapped at the 3′ end of *MIR159b*, with three different sites found from three different clones (Fig. 1*B*). Furthermore, we found two different transcription start sites, 581 and 358 nt upstream of the mature miR159b sequence. Therefore, the largest possible transcript of *MIR159b* was 900 nt long. No introns were present in either the *MIR159a* or *MIR159b* genes.

Using an RNAfold program (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) to predict the secondary structure of the various transcript forms of these two genes, we found considerable variation in their overall structures; however, in each case the stem–loop structures of the pre-miRNAs remained invariant (data not shown). This implied that each transcript isoform could be processed correctly to form a mature miRNA.

***mir159ab* Double Mutant Plants Have Pleiotropic Developmental Defects.** Searching the SIGnAL database (http://signal.salk.edu/cgi-bin/tdnaexpress), we found transfer DNA (T-DNA) insertional mutants belonging to the SAIL collection (15) that lie within *MIR159a* (SAIL_430_F11; designated here as *mir159a*) and *MIR159b* (SAIL_770_G05; designated here as *mir159b*). For *mir159a*, PCR amplification of T-DNA borders determined that the T-DNA was inserted from nucleotide +14 to +51 relative to the transcriptional start site, therefore lying within the primary transcript of the *MIR159a* gene but outside of the stem–loop structure (Fig. 1*A*). The expression of *MIR159a* had been reduced >6-fold in *mir159a* plants but not eliminated (Fig. 1*C*). Because the pre-*MIR159a* structure may still be present, this allele may only represent a hypomorphic mutation. For *mir159b*, the T-DNA has inserted within the region encoding the stem–loop structure (Fig. 1*B*), meaning that any transcript from this allele would be unable to form double-stranded RNA and be processed into a mature miRNA. It is likely that this mutation corresponds to a knockout (null) allele. We examined *MIR159b* expression in *mir159b* and found that the level of transcript containing the miRNA portion was more than seven times higher than wild type (Fig. 1*C*), suggesting that this transcript is not processed, resulting in greater stability and accumulation of this portion of the transcript. Neither *mir159a* nor *mir159b* displayed any obvious morphological traits.

A phylogenetic tree generated with the stem–loop regions of all known *Arabidopsis* and rice *MIR159* genes showed that the *Arabidopsis MIR159a* and *MIR159b* genes were highly similar, suggesting they may be functionally redundant to one another (Fig. 1*E*). In F_2 segregating plants of a cross between *mir159a* and *mir159b*, ~1 plant in 16 (12/197; $\chi^2 = 0.09$; $P > 0.99$) had a distinctive morphological phenotype. By using PCR genotyping, these plants were confirmed to be *mir159ab* mutants, and they failed to accumulate detectable levels of mature miR159 (Fig. 1*D*). Compared with wild-type plants, *mir159ab* growth was stunted, with an altered habit including reduced apical dominance (Fig. 2*A*) and curled (hyponastic) leaves (Fig. 2*B*). Mature siliques of *mir159ab* plants were significantly shorter than those of wild type (Fig. 2*C*), indicating reduced fertility and seed set. Seeds were reduced in size and had an irregular shape (Fig. 2*D*).

***MIR159a* and *MIR159b* Have Similar Expression Patterns Consistent with *MYB33* Repression.** To determine tissue-specific expression of the individual *MIR159* genes, we performed quantitative RT-PCR with gene-specific primers against the pri-*MIR159* transcripts. *MIR159b* was expressed in mature seeds and was induced ≈20-fold after 72 h of imbibition (Fig. 3*A*). Similarly, *MIR159a* was also present in mature seeds and induced (Fig. 3*A*), but to a lesser degree (2- to 3-fold). The timing of induction corresponds to the germination of *Arabidopsis* seeds that occurs between 24 and 48 h of imbibition. *MIR159a* and *MIR159b* were also expressed in the shoot apex region and at a much higher level than *MIR159c* (Fig. 3*B*). This is consistent with *mir159ab* displaying a phenotype and suggests that no further redundancy may exist with respect to miR159-mediated processes in these tissues.

To examine the temporal and spatial expression patterns of the *MIR159* genes, we generated the reporter gene fusions *MIR159a:GUS* and *MIR159b:GUS*, where the regions immediately upstream of the miRNA stem–loop regions were fused to *GUS*. From the examination of multiple transgenic lines for each construct, we found that *MIR159a* and *MIR159b* have near-

Fig. 2. Phenotypic characteristics of *mir159ab* plants. (*A*) The smaller growth stature of *mir159ab* plants. (*B*) Curled leaf phenotype (left) compared with wild-type (right). (*C*) Shorter but fatter fruits in *mir159ab* plants (left). (*D*) Smaller, irregularly shaped seeds of *mir159ab* plants (left) compared with wild type (right).

identical expression patterns (Fig. 4), a fact consistent with their redundancy. They both are strongly expressed in root tips, lateral roots, and the shoot apex region (Fig. 4 *B–D* and *F–H*), the latter possibly providing the rationale for the phenotype of *mir159ab*, because this region is where leaf primordia arise and the architecture of the plant is largely determined (16). Expression

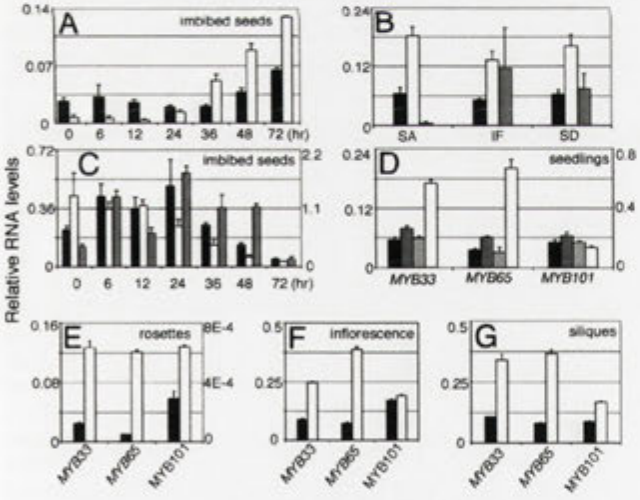

Fig. 3. RNA levels of the *MIR159* genes and their targets. (*A*) Expression of *MIR159a* (black bars) and *MIR159b* (white bars) during seed imbibition. (*B*) Expression of *MIR159a* (black bars), *MIR159b* (white bars), and *MIR159c* (gray bars) in different plant tissues. SA, shoot apical region; IF, inflorescences; SD, 3-day-old imbibed seeds. (*C*) Expression of *MYB33* (black bars), *MYB65* (white bars), and *MYB101* (gray bars) during seed imbibition. (*D*) Expression of the *GAMYB-like* genes in 3-day-old seedlings of wild type (black bars), *mir159a* (dark gray bars), *mir159b* (light gray bars), and *mir159ab* (white bars). (*E*) Expression of the *GAMYB-like* genes in wild-type (black bars) and *mir159ab* (white bars) rosettes. (*F*) Expression of the *GAMYB-like* genes in wild-type (black bars) and *mir159ab* (white bars) inflorescences. (*G*) Expression of the *GAMYB-like* genes in wild-type (black bars) and *mir159ab* (white bars) siliques. Values listed on the right side of graphs correspond to those of *MYB101*.

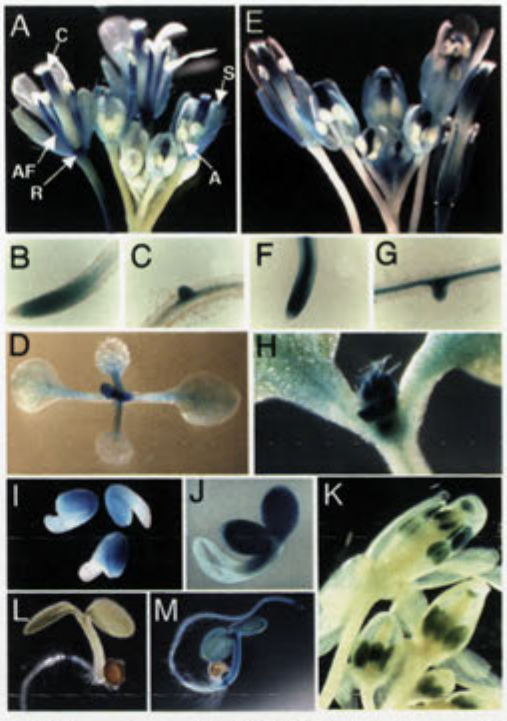

Fig. 4. Spatial expression analysis of *MIR159* and target genes. (*A–D* and *I*) GUS staining of *MIR159a:GUS* transgenic plants in inflorescence (*A*), root tips (*B*), emerging lateral roots (*C*), the shoot apex region (*D*), and seeds (*I*) imbibed for 24 h. (*E–H* and *J*) GUS staining of *MIR159b:GUS* transgenic plants in inflorescence (*E*), roots (*F*), emerging lateral roots (*G*), the shoot apex region (*H*), and 48-h-old seedlings (*J*). Staining of *MIR159b:GUS* lines was generally much stronger than that of *MIR159a:GUS* lines (*K*), *MYB101* promoter:*GUS* in inflorescences (*L*), *MYB33:GUS* in wild-type (*M*), and *MYB33:GUS* in *mir159ab*. AF, anther filament; R, receptacle; S, sepal; A, anther; C, carpels.

is seen in imbibed seeds (Fig. 4 *I* and *J*) and inflorescences (Fig. 4 *A* and *E*), receptacles, anther filaments, sepals, and carpels, with only subtle differences between *MIR159a:GUS* and *MIR159b:GUS*. Expression in these tissues coincides with the regions in which *MYB33* was repressed by miR159, as determined by the comparison of the expression patterns of *MYB33:GUS* and *mMYB33:GUS* (9). The fact that *MIR159:GUS* expression is not seen in anthers accounts for the observation that the only tissue in which *MYB33* is detected in inflorescences is the anthers (9). Therefore, throughout the plant, both *MIR159a* and *MIR159b* are expressed in a temporal and spatial pattern that is totally consistent with the pattern of miR159-mediated *MYB33* repression.

***MYB33* and *MYB65* Expression Levels Are Elevated in *mir159ab*.** We quantified the expression of the seven members of the *GAMYB-like* family that have a conserved motif that miR159 can potentially cleave (9). Of these genes, *MYB33* and *MYB65* are strongly expressed throughout the plant (12, 17). The steady-state levels of these transcripts fall during seed imbibition at the time the transcript levels of *MIR159a/MIR159b* increase (Fig. 3 *A* and *C*). To test whether they are under miR159 control, we measured the transcript levels of *MYB33* and *MYB65* in 3-day-old seedlings of wild-type, *mir159a*, *mir159b*, and *mir159ab* plants (Fig. 3*D*). Whereas expression was mostly unaffected in the single *mir159* mutants, in *mir159ab*, the levels of *MYB33* and *MYB65* were ≈3- and ≈5.4-fold higher than in wild type, respectively, indicating that miR159a and miR159b act redundantly in controlling the expression levels of these genes. These studies were extended to rosettes, inflores-

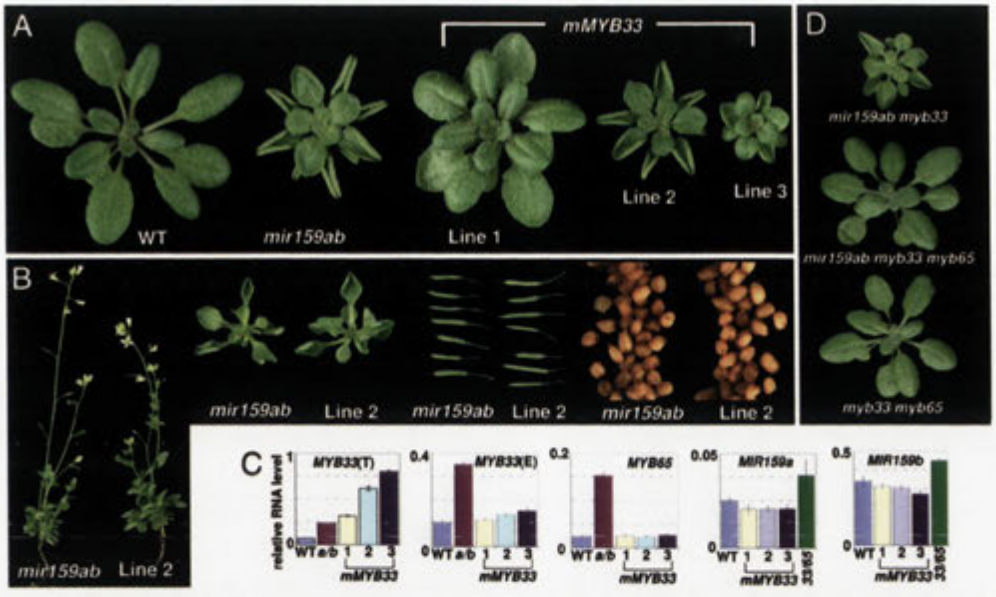

Fig. 5. Phenotypes of *mMYB33* and *mir159ab/myb33/myb65* plants. (*A*) Aerial views of rosettes of 5-week-old plants of wild-type, *mir159ab*, and three *mMYB33* lines grown under short days. (*B*) Aerial views of 3-week-old rosettes of *mir159ab* and *mMYB33* (line 2) grown under long days. Also shown are siliques, seeds, and mature plants from the same lines. (*C*) Quantitative RT-PCR of 6-week-old mature plants was used to determine the relative expression of total [(T)] *MYB33* levels, endogenous [(E)] *MYB33* (using a primer to the miR159 target site that solely amplifies the wild-type *MYB33* allele), *MYB65*, pri-*MIR159a*, and pri-*MIR159b* transcripts. (*D*) Aerial view of rosettes of *mir159ab/myb33*, *mir159ab/myb33/myb65*, and *myb33/myb65*.

cences, and siliques (Fig. 3 *E–G*). In each case, *MYB33* and *MYB65* have considerably higher transcript levels in *mir159ab* plants, demonstrating that these genes are deregulated throughout the plant. This is supported by expression of the *MYB33:GUS* transgene, which was not observable in wild-type seedlings (Fig. 4*L*) but expressed throughout *mir159ab* (Fig. 4*M*).

By contrast, *MYB101* shows little or no difference in expression levels between wild-type and *mir159ab* plants. In siliques and shoot apex regions, *MYB101* levels were only 2-fold higher. For the latter, *MYB101* transcript levels were ≈100-fold lower than *MYB33* and *MYB65*; therefore, this expression level may not be of physiological significance. In inflorescences and 3-day-old seedlings, *MYB101* levels were unchanged. In the case of the inflorescence, an *MYB101* promoter:*GUS* construct (hence without the miR159 target) only shows expression in anthers (Fig. 4*K*). This supports online Affymetrix data (www.genevestigator.ethz.ch/at/) showing that *MYB101* is overwhelmingly expressed in pollen/stamens in the inflorescence (12, 17). This implies that the vast majority of *MYB101* transcripts are not located in the same cell types as that of *MIR159a* and *MIR159b* and is a likely explanation for why *MYB101* levels are not significantly different between wild-type and *mir159ab* plants in inflorescences.

The transcript levels of the other four *GAMYB-like* family members (*MYB81*, *MYB97*, *MYB104*, and *MYB120*) are several orders of magnitude lower than *MYB33* and *MYB65* [supporting information (SI) Fig. 6]. This is consistent with online Affymetrix data, where these genes are expressed primarily in stamens/pollen, with expression either very low or insignificant in any other part of the plant (12, 17). Of the four genes, only *MYB81* has consistently higher transcript levels in *mir159ab* plants, whereas transcript levels of *MYB97* and *MYB120* were in fact lower (SI Fig. 6). In most instances, transcript level differences were only 2- to 3-fold, and this may reflect secondary effects due to the different morphologies of *mir159ab* and wild-type plants rather than miR159 regulation. However, like *MYB101*, these genes are primarily transcribed in anthers, tissues in which miR159a and miR159b appear to be absent, suggesting that they would only make minor contributions to the *mir159ab* phenotype.

***mMYB33* Plants Can Phenocopy *mir159ab*.** Previously, *mMYB33* transgenic plants (the *mMYB33* transgene being under the control of the endogenous *MYB33* promoter) were shown to have curled leaves and stunted growth (9), characteristics similar to that of *mir159ab*. For direct comparison, we grew *mir159ab* alongside three independent *mMYB33* lines that displayed a weak (line 1), intermediate (line 2), and strong (line 3) phenotype and compared their phenotypes throughout development. In all instances, the morphologies of *mir159ab* and *mMYB33* (line 2) plants appear indistinguishable from one another (Fig. 5). This includes the size and shape of the rosettes of plants grown in short days (Fig. 5*A*) or long days (Fig. 5*B*). At bolting, the size and shape of inflorescences and siliques appeared identical as did the seeds they set (Fig. 5*B*). The *MYB33* expression levels in these *mMYB33* lines were positively correlated with the severity of the phenotype (Fig. 5*C*). However, *mir159ab* did not conform to this correlation, reflecting that in addition to *MYB33*, the level of the redundant gene *MYB65* is also higher in *mir159ab* but remains unchanged in the *mMYB33* lines (Fig. 5*C*). Therefore, it is possible that total MYB33/MYB65 activity is at similar levels in *mir159ab* and *mMYB33* (line 2) plants.

We also examined the levels of *MIR159a* and *MIR159b* transcripts in the three *mMYB33* lines, because it has been hypothesized that these genes may be transcriptionally up-regulated by MYB33, resulting in a regulatory feedback loop (10). However, we found no increase in *MIR159a* or *MIR159b* transcript levels in the any of the *mMYB33* lines when compared with wild-type plants (Fig. 5*C*). In addition, we failed to detect evidence of MIR159 down-regulation in the absence of MYB33 and MYB65; *MIR159* levels were not decreased in *myb33/myb65*. Finally, using a primer that discriminated between endogenous and transgenic *MYB33*, we found that the steady-state levels of endogenous *MYB33* levels were not reduced in the *mMYB33* lines (Fig. 5*C*). The fact that both endogenous *MYB33* and *MYB65* levels did not decrease in these *mMYB33* lines again supports the finding that higher miR159 levels are not present in the *mMYB33* lines.

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***myb33* and *myb65* Alleles Suppress the *mir159ab* Phenotype.** All of our data point to *MYB33* and *MYB65* deregulation being predominantly responsible for the *mir159ab* phenotype. To confirm this, we crossed the *myb33* and *myb65* alleles into the *mir159ab* background. A *mir159ab/myb33* triple mutant displayed a milder phenotype than that of *mir159ab*, where growth was less stunted and leaf curling was less severe (Fig. 5*D*). Moreover, in a *mir159ab/myb33/myb65* quadruple mutant, all phenotypic characteristics of *mir159ab* were suppressed, and the mutant appeared to be identical to *myb33/myb65* (Fig. 5*D*). This reversion of the *mir159ab* traits in *mir159ab/myb33/myb65* demonstrates that *MYB33* and *MYB65* are solely responsible for the phenotype exhibited by *mir159ab* plants. Finally, because the phenotype of *mir159ab/myb33* reflects only deregulated *MYB65* activity, this triple mutant confirms that *MYB65* regulates similar processes to that of *MYB33* in the shoot. However, in the *mir159ab* background, *MYB33* and *MYB65* are no longer redundant; their effects have now become additive.

Discussion

Bioinformatics approaches (3), overexpression strategies (12), and isolation of miR159-cleavage products (10, 18) together predicted that the closely related *Arabidopsis MIR159* genes could regulate seven *GAMYB-like* genes. Through the characterization of *Arabidopsis* loss-of-function *mir159* mutants, along with genetic and molecular analyses, we have shown that the predominant role of miR159a and miR159b is to redundantly control just two of these genes, the redundant gene pair of *MYB33* and *MYB65*. This demonstrates a greater functional specificity than previously thought and excludes other regulatory mechanisms, such as targets with low complementarity, that exist in animals.

Currently, there are very few examples of loss-of-function mutants in plant miRNA genes, with only mutations being reported in the miR164 family (19–22). This scarcity has been thought to be due to their small size and/or potential genetic redundancy, because most miRNAs are members of small- to medium-sized gene families (3). Our findings are consistent with the latter, where the single mutants, *mir159a* and *mir159b*, failed to display a phenotype, suggesting that they are fully redundant to one another. This implies that neither *MIR159a* nor *MIR159b* are limiting in controlling target gene expression, and this was shown by the fact that neither *MYB33* nor *MYB65* expression levels increased in the *mir159a* or *mir159b* single mutants (Fig. 3*D*). Furthermore, because only the *mir159ab* double mutant displayed a phenotype under our growth conditions, this indicates that just a single copy of one wild-type allele of either *MIR159a* or *MIR159b* is sufficient to carry out miR159 function, implying that miR159 is produced in a substantial excess.

Of the seven *GAMYB-like* genes, we have demonstrated that the deregulation of the redundant gene pair of *MYB33* and *MYB65* is responsible for the *mir159ab* phenotype. There are several lines of evidence supporting this. *MIR159a:GUS* and *MIR159b:GUS* are expressed exclusively where *MYB33* was being repressed, as determined by analysis of the spatial expression patterns of *MYB33:GUS* and *mMYB33:GUS* (9). For instance, in inflorescences, the only tissue in which *MIR159a:GUS* and *MIR159b:GUS* did not overlap with *mMYB33:GUS* was in anthers, the sole tissues in which *MYB33* is expressed (9). These cotranscriptional domains of miR159a/miR159b and *MYB33* (and presumably *MYB65*) explain why *mir159ab* has global developmental defects, which is in stark contrast to transgenic plants overexpressing a *35S:miR159a* transgene that does not lead to any severe morphological defects other than in anthers (11, 12). Constitutive miR159 expression from a 35S promoter would have little impact, because the transcriptional domains of *MYB33/MYB65* are covered by endogenous miR159a/miR159b. Furthermore, these overlapping transcriptional domains imply that transportation of miR159a or miR159b is not required for them to repress *MYB33*, and this is in agreement with other miRNA systems where *MIRNA* transcription matched precisely to the site of its action (23, 24).

Consistent with these cotranscriptional domains, *MYB33* and *MYB65* transcript levels accumulate to 3- to 10-fold higher throughout *mir159ab* plants. In contrast, the other five *GAMYB-like* genes are predominantly transcribed in anthers and pollen, tissues in which miR159a and miR159b appear to be absent. For *MYB101*, this finding was demonstrated by the anther-specific expression of a *MYB101* promoter:GUS construct. For *MYB104*, the finding is supported by the lack of cleavage products recovered in wild-type *Arabidopsis* (10). Hence, this anther/pollen specificity would explain why the expression of these other five *GAMYB-like* genes do not dramatically increase in *mir159ab* and why *35S:miR159a* plants are male-sterile (11, 12). Furthermore, a pollen-specific gene called *DUO1* that belongs to a different class of MYB transcription factors also contains a functional miR159-binding site; when expressed as an miR159-resistant version under the constitutive 35S promoter, it produces severe developmental defects (18). However, this gene, like the five anther-specific *GAMYB-like* genes, does not contribute to the *mir159ab* phenotype; it appears that miRNA regulation is largely redundant to the transcriptional control of this gene.

The strongly overlapping expression patterns of *mMYB33:GUS* and the *MIR159:GUS* reporter genes suggest that their transcription is controlled by a common regulator, and previously it has been found that they are both induced by gibberellin (11). Furthermore, these overlapping patterns could be explained by a proposed regulatory feedback mechanism where the expression of *MYB33* induces the transcription of miR159 (11). However, the fact that the steady-state transcript levels of *MIR159a* and *MIR159b* were not elevated in the *mMYB33* transgenic lines goes against this possibility. Supporting this, endogenous *MYB33* and *MYB65* steady-state transcript levels were not lower in the *mMYB33* lines, indicating that mature miR159a/miR159b levels have remained unchanged.

By using overexpression strategies and transcriptome analysis, it has been shown that plant miRNAs appear to have only a limited number of targets that they cleave (12). Our loss-of-function strategy suggests that miR159a/miR159b predominantly regulates *MYB33* and *MYB65*, whereas the other predicted targets are predominantly transcribed in tissues where the miRNAs are absent. This scenario is similar to the few examples of miRNA mutants characterized to date. In plants, it was shown that for the *mir164abc* loss-of-function mutant, only two of the targets of miR164 were likely to account for the majority of the phenotypic changes in *mir164abc* plants (22). In animals, although *lin-4* and *let-7* are predicted to regulate many genes, either mutant can be suppressed through the mutation of single target genes (25, 26). One explanation for these observations is that the miRNAs and their targets are transcribed in adjacent but mutually exclusive expression zones, where it is thought that the role of the miRNA is to provide genetic buffering to ensure accuracy to gene-expression programs (27). Similarly, miR159a and miR159b may have a dual role in which they (*i*) cleave transcripts of *MYB33* and *MYB65* in tissues in which they are cotranscribed and (*ii*) ensure that other targets with nonoverlapping transcriptional domains are restricted to those tissues. This may explain the presence of miR159 target sites in the *GAMYB-like* genes that are apparently not targeted by miR159a or miR159b. Alternatively, the presence of these putative target sites may be required for cleavage by miR159c or the closely related miR319 family; recently, they have been shown to have activity against the *GAMYB-like* genes, although they are only very minor regulators of *MYB33* and *MYB65* (18). However, it cannot be ruled out that they are major regulators of the other five *GAMYB-like* genes. It will be of interest to examine what

selective disadvantage *mir159ab/myb33/myb65* plants have now that this highly expressed (5) and highly conserved *MIR159-MYB* regulatory component has been removed.

Materials and Methods

Determination of the pri-*MIR159* Transcripts. To determine the pri-*MIR159* transcripts, 5′ and 3′ RACE reactions were performed with first-strand cDNA synthesized on RNA isolated from imbibed seeds. A GeneRacer kit (Invitrogen Life Technologies, Carlsbad, CA) along with nested PCR primers for *MIR159a* cDNA and *MIR159b* cDNA (SI Table 1) were used to amplify the 5′ and 3′ cDNA ends. The phylogenetic tree of the stem–loop regions was constructed by using ClustalW on the program at www.ebi.ac.uk/Tools/clustalw (28).

Isolation and Genotyping of T-DNA Insertional Mutants. T-DNA mutants were found on the SIGnAL "T-DNA Express" *Arabidopsis* Gene Mapping Tool (29) and were from the Syngenta *Arabidopsis* insertion library (15). Amplification by using the following gene-specific primers (SI Table 1) detected the wild-type alleles: 159a-5 and 159b-3 gave an 884-bp fragment; 159b-5 and 159b-3 gave a 707-bp fragment. To detect the mutant T-DNA alleles, gene-specific primers were combined with the T-DNA-specific primer LB3 in the following combinations: for the *mir159a-1* allele, 159a-5 and LB3 to give a 210-bp fragment and 159b-5 and LB3 to give a 530-bp fragment.

Expression Analysis. RNA was prepared from *Arabidopsis* tissues by using a cetyltrimethylammonium bromide (CTAB) procedure (30). Total RNA (100 μg) was digested with 10 units of RQ1 RNase-free DNase (Promega, Madison, WI) for 15 min at 37°C, then cleaned by using RNeasy Plant columns (Qiagen, Valencia, CA). Five micrograms of this RNA was then used to synthesize cDNA in a 20-μl reaction using SuperScript III (Invitrogen). cDNA was diluted to 100 μl, and then 1 μl was used in 20-μl PCRs in 1× SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis, MO) and 1 μmol of each primer. Specific primers used to quantify each *Arabidopsis* gene are listed in SI Table 1, and the expression of each gene was normalized with *Cyclophilin* (At2g29960). All measurements represent the average of three replicates with error bars representing the standard error of the mean (SEM). For the *MYB* genes, two sets of primers were designed for each gene, either spanning the cleavage site or not. Differences between primer pairs were minimal, and results from only one of the pairs is shown (Fig. 3). For the *MIR159* gene, one primer was located in the stem–loop region and another was located 3′ to the stem loop. All *MIR159* primers fell within the limits of the shortest transcripts as defined in Fig. 1. Analysis of mature miR159 was carried out as described in ref. 31. Oligoprobes for miR159a and U6 were end-labeled with T4 polynucleotide kinase (Promega).

Generation of Binary Vectors and Transgenic Plants. For *pMIR159a:GUS*, upstream sequences (≈1.7 kb) of the *MIR159a* stem loop were PCR-amplified from *Arabidopsis* (Columbia) genomic DNA, with the primers mir159a-13 and mir159a-14 (SI Table 2) and cloned into the HindIII/Sal1 sites of pBI 101.1. For *pMIR159b:GUS*, upstream sequences (≈2.4 kb) of the *MIR159b* stem loop were amplified with the primers mir159b-6 and mir159b-7 and cloned into the HindIII/Xba1 sites of pB1 101.1. For *pMYB101* promoter:*GUS*, the primers 101Pro-5′ and 101Pro-3′ were used to amplify ≈2.3 kb immediately upstream of the start codon of the *MYB101* and cloned into the HindIII/Sal1 sites of pBI 101.1. All amplified DNA was sequenced and confirmed to be correct. Vectors were transformed into *Agrobacterium tumefaciens* (GV3101) and then transformed into *Arabidopsis* by using the floral dip method (32). GUS staining was performed as previously described (9).

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