Modulation of floral development by a gibberellin-regulated microRNA

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Summary

Floral initiation and floral organ development are both regulated by the phytohormone gibberellin (GA). For example, in short-day photoperiods, the *Arabidopsis* floral transition is strongly promoted by GA-mediated activation of the floral meristem-identity gene *LEAFY*. In addition, anther development and pollen microsporogenesis depend on GA-mediated opposition of the function of specific members of the DELLA family of GA-response repressors. We describe the role of a microRNA (miR159) in the regulation of short-day photoperiod flowering time and of anther development. MiR159 directs the cleavage of mRNA encoding GAMYB-related proteins. These proteins are transcription factors that are thought to be involved in the GA-promoted activation of *LEAFY*, and in the regulation of anther development. We show that miR159 levels are regulated by GA via opposition of DELLA function, and that both the sequence of miR159 and the regulation of miR159 levels by DELLA are evolutionarily conserved. Finally, we describe the phenotypic consequences of transgenic over-expression of miR159. Increased levels of miR159 cause a reduction in *LEAFY* transcript levels, delay flowering in short-day photoperiods, and perturb anther development. We propose that miR159 is a phytohormonally regulated homeostatic modulator of GAMYB activity, and hence of GAMYB-dependent developmental processes.

Key words: miR159, GAMYB, Flowering, Photoperiod, Anther

Introduction

Gibberellin (GA) promotes a variety of plant developmental processes by overcoming the repressive effects of the DELLA proteins, a family of nuclear repressors of GA response (Richards et al., 2001). Recent experiments have indicated that GA overcomes repression by DELLA by targeting the DELLA proteins for destruction in the 26*S* proteasome (Fu et al., 2002; McGinnis et al., 2003; Sasaki et al., 2003). One consequence of this is that lack of DELLA proteins can suppress the phenotypic effects of GA deficiency. For example, the gibberellin-deficient *Arabidopsis ga1-3* mutant flowers very late in short-day (SD) photoperiods (Wilson et al., 1992; King et al., 2001), and this phenotype is largely suppressed in *ga1-3* mutants that also lack the DELLA proteins GAI and RGA (Dill and Sun, 2001; King et al., 2001; Cheng et al., 2004). *ga1-3* mutant flowers also exhibit retarded anther development, whilst normal development is restored in *ga1-3* mutants that lack the DELLA proteins RGA, RGL1 and RGL2 (Cheng et al., 2004). Thus GA regulates floral development in a manner analogous to the way in which it promotes growth: the restraining effects of DELLA proteins are overcome by GA, most likely via SCF complex E3 ubiquitin ligase-dependent targeting of DELLAs for destruction in the proteasome (Fu et al., 2002; Sasaki et al., 2003; McGinnis et al., 2003; Harberd, 2003).

First identified in barley aleurone cells (Gubler et al., 1995), GAMYB is a GA-specific transcriptional regulator. GAMYB binds specifically to a GA-response element (GARE) in the 5′ regulatory region of GA-activated genes. Binding of GAMYB to the GARE activates the transcription of genes encoding the hydrolytic enzymes that release stored nutrients from the endosperm during seed germination (Cercos et al., 1999; Gubler et al., 1999). Expression of *GAMYB* (the gene encoding GAMYB) is itself up-regulated by GA (Gubler et al., 1995). In addition, constitutive expression of GAMYB mimics the activating effects of GA application on the transcription of genes encoding hydrolytic enzymes, suggesting that GAMYB plays an important role in the GA signalling pathway in aleurone cells (Cercos et al., 1999). Recent experiments have shown that transgenic overexpression of GAMYB perturbs the development of anthers, indicating that GAMYB can function in cells other than those of the aleurone layer (Murray et al., 2003). Furthermore, loss-of-function mutations in the rice *GAMYB* gene have recently been shown to abolish GA-mediated induction of the aleurone hydrolase α-amylase, to retard the growth and development of stamens and anthers, and to impair microsporogensis (Kaneko et al., 2004). These latter observations provide genetic proof of the involvement of GAMYB in both aleurone physiology and floral organ development.

Genes encoding proteins closely related in sequence to the barley and rice GAMYB have been identified in *Arabidopsis* [*AtMYB33*, *AtMYB65* and *AtMYB101* (Gocal et al., 2001)]. Amongst these AtGAMYBs, AtMYB33 is the most similar to the barley GAMYB (Gocal et al., 2001). The AtGAMYBs have

been suggested to be involved in the GA-mediated promotion of flowering in SDs, by activation of the floral meristem identity gene *LEAFY* (Blázquez et al., 1998; Blázquez and Weigel, 2000; Gocal et al., 2001). Activation of *LEAFY* is known to be important in this process because transgenic over-expression of *LEAFY* restores a wild-type flowering time to *ga1-3* plants grown in SDs (Blázquez et al., 1998). AtMYB33 binds in vitro to a GARE in the *LEAFY* promoter (a GARE that has been shown to be essential for GA induction of *LEAFY*) and fails to bind to non-functional mutant derivatives of this element. This suggests that GAMYB plays a key role in *LEAFY* activation (Blázquez and Weigel, 2000; Gocal et al., 2001). Another floral integrator gene, *SOC1*, has recently been shown to play an important role in the GA promotion of flowering in SD photoperiods (Moon et al., 2003), and it has been suggested that GAMYB and SOC1 both act upstream of *LEAFY* (Lee et al., 2000; Mouradov et al., 2002; Yu et al., 2002).

To further our understanding of the role of the AtGAMYBs in the regulation of floral development we took advantage of the recent identification of a microRNA (miR159) exhibiting substantial sequence homology with mRNAs encoding AtMYB33, AtMYB65 and AtMYB101 (Gocal et al., 2001; Reinhart et al., 2002). MicroRNAs are single-stranded RNA molecules of 20-22 nucleotides that can cause complementarity-dependent cleavage (e.g. Llave et al., 2002a) or translational inhibition (Chen, 2004; Aukerman and Sakai, 2003) of target mRNA molecules. More than one hundred distinct small RNAs have been identified in plants, although only a minority of these are microRNAs (Llave et al., 2002b; Reinhart et al., 2002). Most of the predicted targets of these microRNAs are members of transcription factor gene families involved in developmental patterning or cell differentiation (Rhoades et al., 2002; Kidner and Martienssen, 2003). In this paper we describe experiments showing that miR159 directs the cleavage of AtMYB33-encoding transcripts (see also Palatnik et al., 2003). We also show that miR159 sequence is evolutionarily conserved, and that miR159 levels are GA-regulated via a mechanism that is evolutionarily conserved between *Arabidopsis* and barley and that is dependent on the GA-promoted opposition of the function of DELLA protein GA-response repressors. To further investigate the role of miR159 in vivo, we generated transgenic *Arabidopsis* plants over-expressing miR159 and examined the floral developmental phenotypes of these plants. We found that elevated expression of miR159 resulted in a delay in flowering in SD photoperiods that was associated with a reduction in the levels of *LEAFY* transcripts. In addition, previous experiments in barley and rice have identified a role for GAMYB in anther development (Murray et al., 2003; Kaneko et al., 2004). We found that elevated levels of miR159 perturbed anther development and caused a consequent reduction in floral fertility. These observations suggest that miR159 modulates GA-mediated developmental regulation via its effects on GAMYB activity.

Materials and methods

Plant material and growth conditions

Arabidopsis experiments used the Landsberg *erecta* laboratory strain of *Arabidopsis thaliana*. The *ga1-3*, *gai* and *ga1-3 gai-t6 rga-24* mutations were as previously described (Achard et al., 2003). The plants were grown in a growth cabinet in long days (LDs; 22°C, 16-hours photoperiod) or in short days (SDs; 22°C, 10-hour photoperiod) as indicated. Barley (*Hordeum vulgare*) floral tissues were obtained from 7-week-old greenhouse-grown plants (16 hour photoperiod). Tobacco (*Nicotiana benthamiana*) leaves and floral tissues were harvested from 5-week-old greenhouse-grown plants (16-hour photoperiod).

Plasmids and construction

The *35S:MYC-MYB33* construct contained a copy of the *AtMYB33* (accession number AF411969) coding sequence. The cDNA was amplified from total RNA by RT-PCR using gene-specific primers (*AtMYB33*: GCATGTTGAGGCACTTGAGTGGAGTC and TCTGGAATCATAACAGGTAATGTCGG) and cloned into the p35S-2 vector (P. Mullineaux, John Innes Centre). Three myc epitope tags were inserted in phase, upstream of the *AtMYB33* cDNA. After digestion, an *Eco*RV fragment containing the construction *35S:MYC-MYB33* was inserted into the plant transformation vector pGreen0029 (P. Mullineaux, John Innes Centre). A mutated version of the *AtMYB33* transgene (*35S:MYC-mMYB33*) was generated by PCR using the Quick Change XL site-directed mutagenesis kit (Stratagene). The mutated *mMYB33* primers used were as follows: GGCAGTGAAGCTGGAATTGCCAAGCTTTCAATATTCAGAAACAAC and GTTGTTTCTGAATATTGAAAGCTTGGCAATTCCAGCTTCACTGCC. The nucleotide sequence of the *mMYB33* cDNA was as described by Palatnik et al. (Palatnik et al., 2003). The *35S:miR159a* construct contained a copy of the precursor of miR159 isoform a (Fig. 1A). A fragment of 228 bp (containing the precursor of 182 bp) was amplified from genomic DNA by PCR using specific primers (miR159a: GAGAAGGTGAAAGAAGATGTAGAGCTCCC and CGATAGATCTTGATCTGACGATGGAAG). These primers were designed to anneal specifically to genomic DNA on either side of the predicted miR159a hairpin precursor. The amplified fragment was then cloned into the p35S-2 vector, and an *Eco*RV fragment containing the construct *35S:miR159a* was then inserted into the pGreen0029 vector. The *35S:GFP* construct was made similarly using GFP-specific primers: CGGCACGACTTCTTCAAGAGCGC and GTATTCCAACTTGTGGCCGAGG. The resulting constructs were introduced into *Agrobacterium tumefaciens* strain GV3101.

Infiltration of *Agrobacterium tumefaciens* into *N. benthamiana*

Agro-infiltration was performed in *Nicotiana benthamiana* leaves as described previously (Llave et al., 2002a). *35S:miR159a*, *35S:MYC-MYB33*, *35S:MYC-mMYB33* and *35S:GFP* were introduced into *A. tumefaciens* and the bacteria injected into *N. benthamiana* leaves with a syringe. For co-injections of two or three different constructs, bacteria were resuspended in infiltration medium (0.5× Murashige and Skoog salts, 5% sucrose, 0.5 g/l Mes) at OD_{600}=1, and incubated for 3 hours at room temperature with 150 μM acetosyringone. Zones of infiltration were harvested at 4 and 5 days after injection for RNA and protein isolations. The *35S:GFP* construct was used as a control for the co-Agro-infiltration.

Transformation of *Arabidopsis thaliana*

Transformation was performed by floral dipping (Clough and Bent, 1998). *A. tumefaciens* containing the appropriate constructs were resuspended in infiltration buffer (0.5× Murashige and Skoog salts, 5% sucrose, 0.5 g/l Mes, 0.05% Silwet L77) at OD_{600}=1. Transformants were selected by resistance to 50 mg/l of kanamycin sulphate. Plants in Fig. 5A-C, Figs 6 and 7 were homozygous for the *35S:miR159a* transgene (T_2 generation); plants in Fig. 5D,E were from segregating populations (self-pollination progeny of *35S:miR159a* primary transformants).

Nucleic acid isolation and blot analysis

Total RNA was extracted using the Trizol reagent (Gibco-BRL) and

resuspended in 50% formamide. Low molecular mass and high molecular mass RNAs were isolated by three successive precipitations in PEG_{8000}/NaCl as previously described (Hamilton et al., 2002). 20 μg high molecular mass RNA was separated in a 1% denaturing agarose gel and subjected to blot-hybridisation analysis. Radiolabelled probes were prepared from a 200 bp 3′-end *MYB33* fragment or from full-length *LEAFY, SOC1* and *ELF4a* open reading frames via random priming reactions (Roche) in the presence of [α^{32}P]dCTP. 20 μg low molecular mass RNA was separated in a 15% denaturing polyacrylamide gel and subjected to blot-hybridization analysis. Small RNA (miR159a: TAGAGCTCCCTTCAATCCA-AAGA; miR167: TAGATCATGCTGGCAGCTTCA) probes were labelled with T4 kinase (Gibco-BRL) in the presence of [γ^{32}P]ATP. Since miR159 hybridises with miR-JAW [a microRNA that is more divergent from miR159 than each of the miR159 isoforms are from each other; Fig. 1A (Palatnik et al., 2003)], it is likely that the miR159a probe used in our experiments hybridises with all three isoforms of miR159. Each RNA blot analysis was repeated at least twice.

Protein isolation and analysis

Agro-infiltrated tobacco leaves were harvested and frozen in liquid nitrogen. Total protein was extracted and quantified as described by Achard et al. (Achard et al., 2003). 20 μg of proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection of the MYC-MYB33 fusion protein was performed using a 2000-fold dilution of anti-cMYC polyclonal antibodies from goat (Santa Cruz Biotechnology, CA) and a 2000-fold dilution of peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology, CA). Immunodetection of GFP was performed using a 2500-fold dilution of anti-GFP monoclonal antibodies from mouse (Chemicon International, Temecula, CA, USA) and a 5000-fold dilution of peroxidase-conjugated goat anti-mouse IgG (SouthernBiotech). Signals were detected by chemiluminescence using the ECL Western Blotting Analysis System (Amersham Biosciences).

Results

MiR159 is a post-transcriptional regulator of GAMYB

MiR159 is a microRNA of 21 nucleotides in length derived from a precursor molecule of 182 nucleotides (Reinhart et al., 2002). In *Arabidopsis*, miR159 is present in three isoforms (Fig. 1A). MiR159 sequence is of near-perfect complementarity to an internal sequence of five *Arabidopsis* mRNAs encoding three GAMYB-like proteins (AtMYB33, AtMYB65 and AtMYB101) and two additional MYB proteins (Fig. 1B). The *Arabidopsis* miR159 sequence is also closely complementary to an internal sequence of several *GAMYB* mRNAs from barley (*Hordeum vulgare; HvGAMYB*), *Oryza sativa*, *Avena sativa* and *Lolium temulentum* (Fig. 1B). AtMYB33, AtMYB65 and AtMYB101 share substantial homology with HvGAMYB in an N-terminal region corresponding to an R2R3 repeat DNA-binding domain (Fig. 1C). These proteins also contain three additional conserved motifs (Box 1, Box 2 and Box 3) that are characteristic of GAMYB (Gocal et al., 2001). The zone that is complementary between miR159 and the GAMYB-encoding mRNAs is located about 100 nucleotides upstream of the sequence encoding Box 2 (Fig. 1C).

MicroRNAs can regulate gene expression by directing cleavage or by inhibition of translation of target transcripts (Llave et al., 2002a; Chen, 2004; Aukerman and Sakai, 2003). We tested if miR159 directs the cleavage of *GAMYB*-like mRNAs (see also Palatnik et al., 2003) using an *Agrobacterium*-mediated delivery system to co-express

A

miR159a	tagagctcccttcaatccaaa
miR159b	aagagctcccttcaatccaaa
miR159c	aggagctcccttcaatccaaa

B

Gene	Sequence	Mismatches	Function	Accession
Arabidopsis thaliana				
AtMYB33	tggagctcccttcattccaat	3	GAMYB	AF411969 / At5g06100
AtMYB65	tggagctcccttcattccaat	3	GAMYB	AF048840 / At3g11440
AtMYB101	tagagcttccttcaaaccaaa	2	GAMYB	AF411970 / At2g32460
AtMYB104	tggagctcccttcattccaag	3	MYB	U26934 / At2g26950
AtMYB81	tcgagttcccttcattccaat	4	MYB	AF062911 / At2g26960
Hordeum vulgare				
HvGAMYB	tggagctcccttcactccaag	3	GAMYB	X87690
Oryza sativa				
OsGAMYB	tggagctcccttcactccaag	3	GAMYB	X98355
Avena sativa				
AvGAMYB	tggagctcccttcactccaag	3	GAMYB	AJ133638
Lolium temulentum				
LtGAMYB	tggagctcccttcactccaag	3	GAMYB	AF114162

C

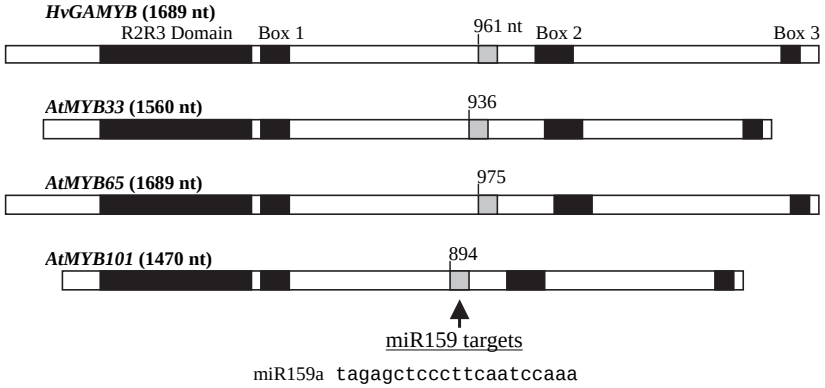

Fig. 1. MiR159 and *GAMYB* mRNA target sequences are evolutionarily conserved. (A) Sequence similarity between three *Arabidopsis* miR159 isoforms. The nucleotide mismatches are in grey. (B) Sequence similarity between miR159a and internal sequences of putative target genes. Nucleotide mismatches between the internal sequences and miR159a are in grey, and the total number of mismatches is indicated after each sequence. Function and accession number for each putative target is indicated on the right. (C) Location of the complementary sequence between *GAMYB* genes and miR159. Regions of similarity between *Arabidopsis* and *H. vulgare GAMYB* genes are shown in black. The region of sequence that is complementary between miR159a and the *GAMYB* genes is shown in grey.

miR159 and *MYB33* target mRNA in *N. benthamiana* leaf tissue (Llave et al., 2002a) (Fig. 2). Four constructs (*35S:miR159a*, *35S:MYC-MYB33*, *35S:MYC-mMYB33* and *35S:GFP*) were inoculated and expressed (Fig. 2A). Whilst basal levels of endogenous miR159 were detected in tissues lacking the *35S:miR159a* construct, increased levels of miR159 were detected in tobacco leaves inoculated with this construct, indicating that over-expression and processing of transgenically derived miR159a was successfully achieved (Fig. 2B). Expression of the *35S:MYC-MYB33* construct resulted in detectable levels of *MYC-MYB33* transcripts (*MYC-MYB33a*; Fig. 2B). Co-expression of *35S:MYC-MYB33* with *35S:miR159a* resulted in loss of the full length *MYC-MYB33a* transcript form, and the appearance of detectable levels of a smaller transcript (*3′-MYB33b*) that hybridized to the *MYB33* probe (a 3′ probe fragment, see Materials and methods; Fig. 2B). We attribute this smaller fragment to internal cleavage of the *MYC-MYB33* mRNA directed by miR159 (as previously suggested by 5′ RACE-PCR experiments; Palatnik et al., 2003). Consistent with this attribution, co-expression of *35S:MYC-mMYB33* (encoding a *MYC-mMYB33* transcript that carries an altered miR159 binding site; see Materials and methods) with *35S:miR159a* resulted in detectable transcripts of the *MYC-MYB33a* size, but not of the *3′-MYB33b* size.

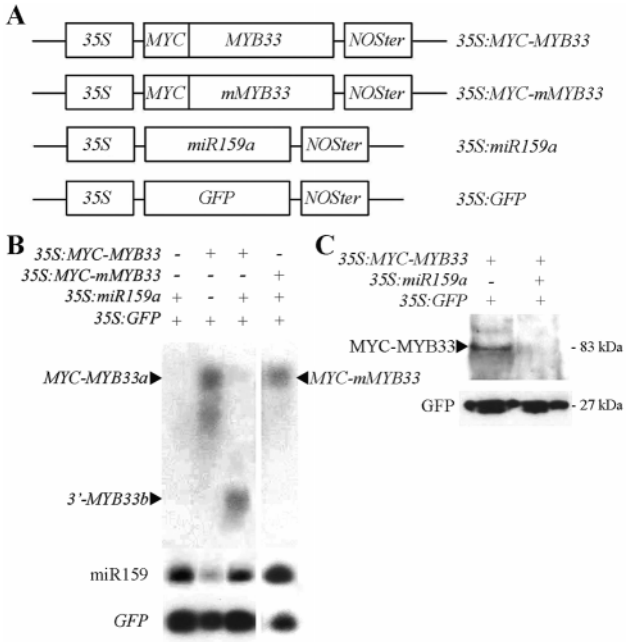

Fig. 2. MiR159 directs the cleavage of *MYB33* transcripts and regulates MYB33 levels. (A) Constructs specifying miR159a, *MYC-MYB33* and *MYC-mMYB33* RNAs and driven by the 35S promoter. *35S:GFP* was used as control for co-Agro-inoculation. (B) RNA gel-blot analysis of miR159, *MYC-MYB33* and *MYC-mMYB33* RNA forms (full length *MYC-MYB33a* and cleaved *3′-MYB33b* transcripts) in *N. benthamiana* leaves co-Agro-inoculated with different combinations of *35S:miR159a*, *35S:MYC-MYB33*, *35S:MYC-mMYB33* and *35S:GFP* as indicated. (C) Immunoblot analysis of total proteins (20 μg) from *N. benthamiana* leaves co-Agro-inoculated as in B. Goat anti-myc antiserum and a peroxidase-conjugated anti-goat IgG were used as primary and secondary antibodies, respectively. The lower panel shows a GFP immunoblot using the same extracts (loading control).

Subsequently, we tested whether miR159 causes a reduction in the level of MYC-MYB33 fusion protein (Fig. 2C). A peptide of ~83 kDa was recognised by MYC antibodies in a sample from leaves expressing *35S:MYC-MYB33* but not in a sample co-expressing *35S:miR159a* and *35S:MYC-MYB33* (Fig. 2C). This peptide was of the size expected for the MYC-MYB33 fusion protein encoded by *35S:MYC-MYB33*. Taken together, these RNA gel-blot and immunoblot analyses indicate that miR159 can direct the cleavage of transcripts encoding GAMYB, thus down-regulating GAMYB levels.

MiR159 is an evolutionarily conserved sequence

We next investigated the distribution of miR159 in *Arabidopsis*, tobacco (*Nicotiana benthamiana*) and barley. In *Arabidopsis*, miR159 accumulated predominantly in young seedlings and flowers, was less abundant in rosette leaves, cauline leaves or siliques, and was undetectable in roots (Fig. 3). Interestingly, miR159 was also clearly detectable in tobacco and barley. As in *Arabidopsis*, miR159 accumulated predominantly in the inflorescence and floral tissues in these species (Fig. 3). These observations indicate that the sequence and developmental regulation of miR159 is highly conserved, despite the considerable evolutionary distance that separates *Arabidopsis*, tobacco and barley from their last common ancestor.

GA enhances miR159 levels by opposing DELLA function

Since GAMYB functions in GA-mediated gene regulation, we next investigated the possibility that GA might itself regulate miR159 levels. The *Arabidopsis ga1-3* mutant is GA deficient and exhibits a dwarf, dark-green phenotype (Koornneef and van der Veen, 1980; King et al., 2001). MiR159 was less abundant in flowers of *ga1-3* than in wild-type flowers (Fig. 4A). MiR159 levels in GA-treated *ga1-3* plants were similar to those in GA-treated wild-type plants, and higher than in untreated wild-type controls (Fig. 4A). These data indicate that miR159 levels are GA regulated.

GA-regulated processes are usually controlled via the effects of GA on DELLA protein function (Richards et al., 2001). In many cases, DELLA proteins act as repressors of GA-response, and GA acts by targeting the DELLA proteins for destruction in the proteasome (Fu et al., 2002). We therefore tested whether GA enhances miR159 levels by overcoming the

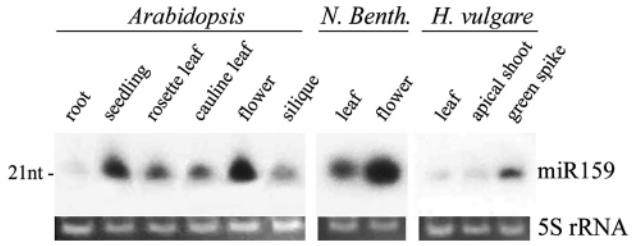

Fig. 3. MiR159 levels vary in different tissues. RNA gel-blot analysis of miR159 from *Arabidopsis* root, 5 day-old seedling, rosette leaf, cauline leaf, flower and silique tissues; *N. benthamiana* leaf and flower tissues; barley (*Hordeum vulgare*) leaf, apical shoot and green spike (inflorescence) tissues. 5S rRNA was used as RNA control (shown in ethidium bromide-stained gel).

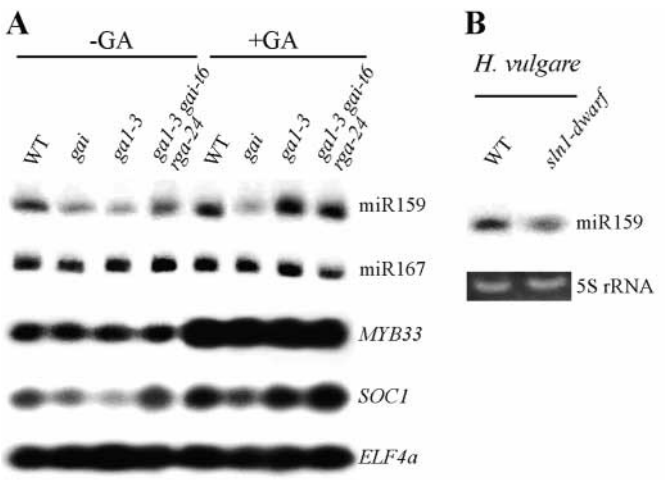

Fig. 4. GA regulates miR159 levels via the DELLA-dependent GA signalling pathway. (A) RNA gel-blot analysis of miR159, *MYB33* and *SOC1* from wild-type (WT), *gai*, *ga1-3* and *ga1-3 gai-t6 rga-24* plants grown in long days (LD) in the presence (+GA) or absence (–GA) of gibberellin (100 μM GA_3 sprayed twice per week). The blots were stripped and re-analysed with miR167 and *ELF4a* probes (RNA controls). (B) RNA gel-blot analysis of miR159 levels in wild-type and *sln1-dwarf* mutant barley (*Hordeum vulgare*). 5S rRNA was used as RNA control.

effects of DELLA proteins. Firstly, we found that the level of miR159 was reduced in *gai* mutant plants (Fig. 4A). The *gai* mutant shares many phenotypic characteristics with *ga1-3*, except that the *gai* phenotype cannot be restored to normal with GA (Koornneef et al., 1985; Peng et al., 1997). *gai* encodes a dominant altered-function mutant GAI protein (gai) that is resistant to the opposing effects of GA and confers a reduced GA response (Peng et al., 1997; Richards et al., 2001). Consistent with this we found that the reduced miR159 levels in *gai* were not restored to normal by GA. Secondly, we investigated the effect of lack of the DELLA proteins GAI and RGA on miR159 levels in *ga1-3*. Previous experiments have shown that various aspects of the *ga1-3* phenotype are suppressed in plants lacking GAI and RGA (Dill and Sun, 2001; King et al., 2001; Fu and Harberd, 2003; Cheng et al., 2004). We found that *ga1-3* plants lacking GAI and RGA (*ga1-3 gai-t6 rga-24*) had miR159 levels that were not detectably different from those of wild-type plants (Fig. 4A). Taken together, these observations indicate that the DELLA proteins GAI and RGA repress miR159 levels, and that GA promotes miR159 levels by overcoming DELLA-mediated repression.

We next compared the regulation of miR159 level in *Arabidopsis* with its regulation in barley. Barley *SLN1* encodes a DELLA protein (Chandler et al., 2002; Fu et al., 2002), and the dominant dwarfing *sln1-dwarf* allele encodes a mutant protein that is altered in the highly conserved DELLA domain (X. Fu, D. E. Richards and N.P.H., unpublished) (see also Peng et al., 1999; Chandler et al., 2002; Itoh et al., 2002). The effect of this mutation is analogous to that of the *Arabidopsis gai* allele. We found that miR159 levels were reduced in *sln1-dwarf* compared to *SLN* barley (Fig. 4B). Thus, GA-DELLA system regulation of miR159 levels is evolutionarily conserved across the divided lineage that separates *Arabidopsis* and barley.

Exogenous GA enhances *MYB33* transcript levels

We also compared the levels of *MYB33* transcripts in the *Arabidopsis* GA-signalling mutant lines. *MYB33* transcript levels were not substantially different in wild type, *ga1-3*, *gai* or *ga1-3 gai-t6 rga-24* (grown in the absence of exogenous GA; Fig. 4A). However, *MYB33* transcript levels were consistently higher in all of these genotypes when grown in the presence of exogenous GA (Fig. 4A). Since *MYB33* is the target of miR159, it might have been expected that there would be an inverse correlation between miRNA and *MYB33* transcript levels. Yet whilst miR159 levels were reduced in *ga1-3* (and restored by GA or lack of GAI and RGA) there were no detectable differences in *MYB33* transcript levels in these various mutant lines. This apparent paradox is considered further in the Discussion.

MiR159 level modulates photoperiodic control of the floral transition

Recent studies suggest that GA might regulate flowering via GAMYB-dependent activation of the floral meristem identity gene *LEAFY* (Blázquez and Weigel, 2000; Gocal et al., 2001). In *Arabidopsis*, the GA pathway has a stronger effect on flowering in SD than it does in LD photoperiods (Wilson et al., 1992; Mouradov et al., 2002; Moon et al., 2003). We therefore investigated the effect of elevated expression of miR159 on flowering time in SDs. Four-week-old SD-grown transgenic plants over-expressing miR159a (homozygous for *35S:miR159a*) had leaves that were smaller and rounder than wild-type control plants (Fig. 5A). In addition, SD-grown *35S:miR159a* plants exhibited a delay in flowering time (Fig. 5B). Wild-type plants bolted then flowered at day 39 (±1.12) whereas transgenic plants flowered at day 53 (±4.66) (Fig. 5C). A delay in flowering time (of varying severity) was observed in plants of ten additional independent transgenic lines (each homozygous for *35S:miR159a*), and was heritable in the progeny of these plants (data not shown). *35S:miR159a* homozygotes also produced more rosette leaves than did wild-type controls when grown in SDs (37±2.63 against 23±2.38 for wild type; Fig. 5C). In LD photoperiods the effect of *35S:miR159a* was much less than in SDs, with LD-grown plants over-expressing miR159a flowering at approximately the same time as wild-type plants (actually 1 day later; data not shown).

In subsequent experiments, segregation of the late-flowering phenotype was followed in two independent transgenic families that were segregating for *35S:miR159a*. Only those plants over-expressing miR159a exhibited late flowering in SDs, whilst segregants expressing normal levels of miR159 flowered at the same time as non-transgenic wild-type controls (Fig. 5D). We next determined the levels of *MYB33* transcripts, the targets of miR159, in these plants. *MYB33* transcripts were detected at substantially reduced levels in plants over-expressing miR159a (and exhibiting a late flowering phenotype), but were not reduced in segregating plants that expressed normal levels of miR159 (Fig. 5E). In addition, the pattern of *MYB33* transcript accumulation correlated with that of *LEAFY* transcripts. *LEAFY* transcript levels were reduced in SD-grown *35S:miR159a* plants, but not in segregants expressing normal levels of miR159 (Fig. 5E). Taken together, these observations suggest that miR159 directs the cleavage of *MYB33* transcripts, that the resultant reduction in MYB33 level reduces *LEAFY* activity, and that the reduction in *LEAFY* activity delays SD flowering.

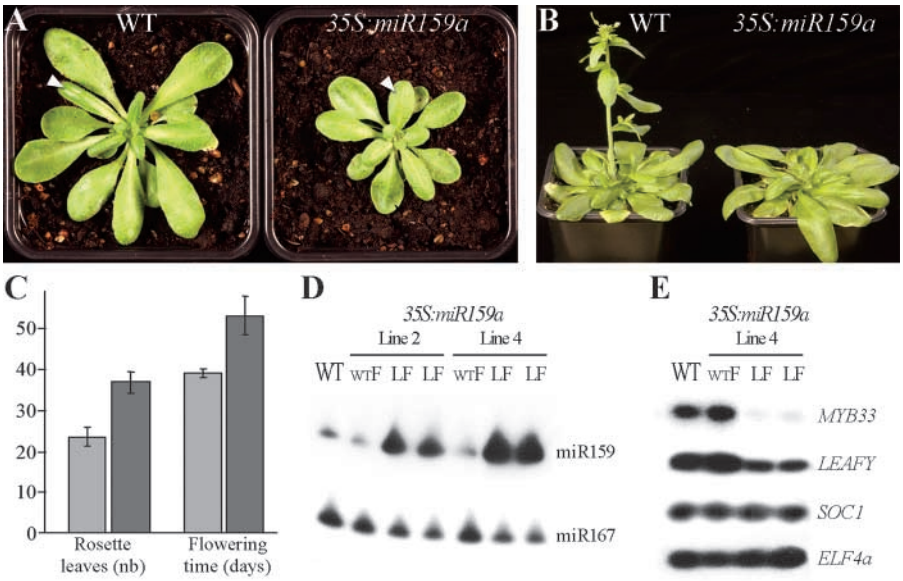

Fig. 5. Overexpression of miR159a delays the floral transition in short days (SDs). (A) wild-type (WT) and *35S:miR159a* homozygotes grown for 4 weeks in SDs. The arrow indicates a new rosette leaf. (B) WT and *35S:miR159a* homozygotes grown for 6 weeks in short days. (C) Means of the number of total leaves produced before flowering (nb), and of the number of days to flower in SD conditions (±s.e.; n>10), are shown. Wild type, light grey bars; *35S:miR159a* homozygotes, dark grey bars. (D) RNA gel-blot analysis of miR159 levels in 4-week-old wild-type and *35S:miR159a* leaves. Lines 2 and 4 are clonally independent (T_1) transgenic lines that segregate *35S:miR159a*. WTF, segregants lacking the transgene, flowered at the same time as wild type (non-transgenic control); LF, late flowering segregants carrying *35S:miR159a*. (E) RNA gel-blot analysis of *MYB33*, *LEAFY* and *SOC1* mRNA performed on the same plant material as in D. *ELF4a* transcript was used as a RNA sample control.

Recent experiments have shown that *SUPPRESOR OF OVEREXPRESSION OF CO1* (*SOC1*) is also an important component of the GA-dependent flowering pathway and that integration via *SOC1* is necessary for flowering in SDs (Moon et al., 2003). We therefore investigated the relationships between the GA-DELLA system, miR159, *MYB33* and *SOC1*. To begin with, we tested whether the DELLA proteins GAI and RGA regulate *SOC1* expression. *SOC1* transcripts were less abundant in GA-deficient *ga1-3* plants than in wild-type plants. In contrast, similar levels of *SOC1* transcripts were observed in wild-type and *ga1-3* plants treated with exogenous GA (Fig. 4A) (see also Moon et al., 2003). Two further observations indicated that GA regulates *SOC1* transcript levels via opposition of DELLA repression. First, *SOC1* transcript levels were reduced in *gai* mutant plants (compared to wild type) and not restored to wild-type levels by exogenous GA (Fig. 4A). Thus the constitutively repressing mutant gai protein confers a reduction in *SOC1* transcript levels. Secondly, *SOC1* transcript levels in *ga1-3* plants lacking GAI and RGA (*ga1-3 gai-t6 rga-24*) were little different to those in wild-type plants (Fig. 4A). Thus lack of GAI and RGA suppressed the effect of *ga1-3* on *SOC1* transcript levels, indicating that GA regulates *SOC1* levels (and thus flowering time in SD) by opposition of GAI and RGA function.

How does GA-pathway regulation of SD flowering via *SOC1* relate to GA-pathway regulation of flowering via miR159/*MYB33*? To answer this question we determined *SOC1* transcript levels in plants from a family segregating for *35S:miR159a*. Late flowering plants contained *SOC1* transcript levels that were similar to those of non-transgenic wild-type plants (Fig. 5E). Thus an increase in miR159 level has no downstream effect on *SOC1* transcript levels, demonstrating that the *SOC1*- and miR159/*MYB33*-dependent signalling pathways through which GA regulates flowering in SD act independently of one another.

Elevated miR159 levels affect anther development

GA and GAMYB are known to be involved in the regulation of anther development (Murray et al., 2003; Cheng et al., 2004; Kaneko et al., 2004). We next investigated the possible role of miR159 in anther development. LD-grown *35S:miR159a* homozygotes had smaller cauline leaves than wild-type controls (Fig. 6A). In addition, *35S:miR159a* plants had short, sterile siliques (Fig. 6C,D). *35S:miR159a* siliques were ~40% of the length of wild-type siliques and contained no seeds (Fig. 6B; eventually a few fertile siliques were obtained in some plants). In other respects the development of *35S:miR159a* homozygotes was indistinguishable from that of wild type.

In order to determine the cause of the infertility, the floral development of *35S:miR159a* homozygotes was compared with that of wild-type controls. Except for the anthers, the floral organs of *35S:miR159a* plants appeared relatively normal. However, the increase in miR159 levels (and decrease in *MYB33* transcript levels; Fig. 6E) characteristic of *35S:miR159a* plants was associated with a progressive increase in the size of anthers, and with a darkening of anther colour (Fig. 7A-D). In addition, the *35S:miR159a* anthers failed to release pollen (Fig. 7D). Since seed-bearing siliques were recovered following cross-fertilization of *35S:miR159a* with wild-type pollen (data not shown) it was clear that the infertility of the *35S:miR159a* plants was due to male sterility resulting from failure to release pollen. The anther phenotype characteristic of the *35S:miR159a* plants was visible from floral developmental stage 10 (Fig. 7E) and was clear until stage 12 (Fig. 7F). Subsequently, the anthers became increasingly dark in colour (Fig. 7B,D). The final stamen lengths in wild-type and *35S:miR159a* flowers were indistinguishable (data not shown).

Discussion

Although the recent discoveries of small RNA species in the cells of diverse organisms have generated much excitement, there are still relatively few cases where small RNAs have been definitively linked to specific plant developmental functions (Chen, 2004; Aukerman and Sakai, 2003; Emery et al., 2003; Palatnik et al., 2003). In this paper we describe experiments designed to investigate the developmental function of miR159.

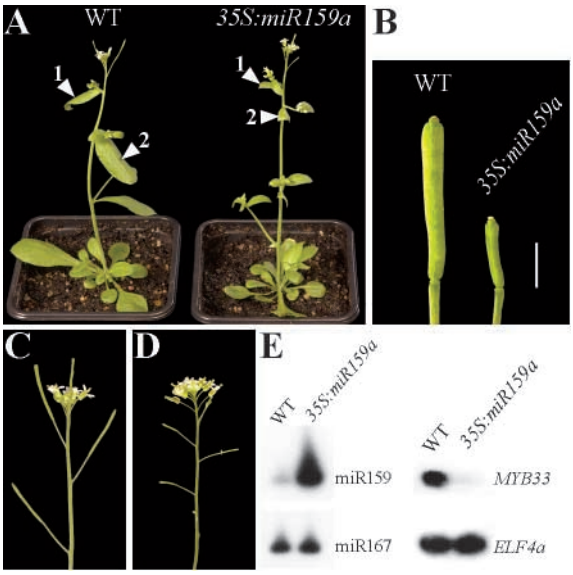

Fig. 6. *35S:miR159a* plants have reduced fertility. (A) Wild type and *35S:miR159a* homozygotes grown for 4 weeks in a LD photoperiod (16 hours light). The arrows indicate the two last cauline leaves formed. (B) Siliques of wild type and *35S:miR159a* homozygotes. Scale bar: 3 mm. (C) Wild-type inflorescence. (D) Inflorescence of *35S:miR159a* homozygote. (E) RNA gel-blot analysis of miR159 and *MYB33* from wild-type and *35S:miR159a* flowers.

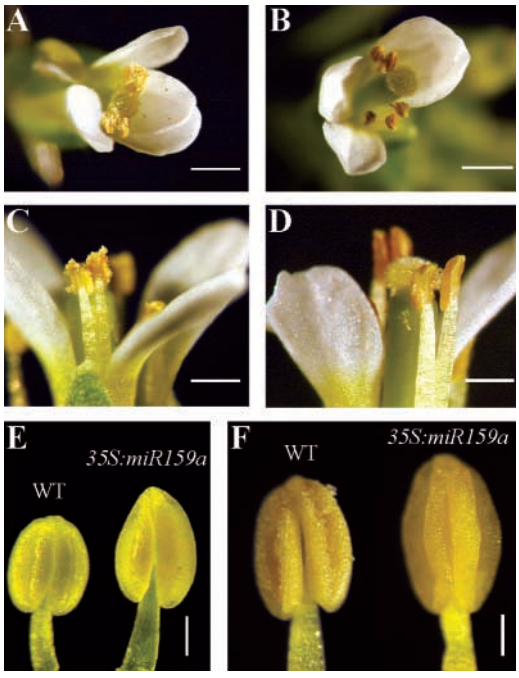

Fig. 7. Overexpression of miR159a affects anther development. (A,C) Wild-type flowers. (B,D) Flowers of *35S:miR159a* homozygotes. (E) Anthers from wild-type and *35S:miR159a* homozygote [taken from flowers at floral development stage 10 (Cheng et al., 2004)]. (F) Anthers from wild-type and *35S:miR159a* homozygotes (from flowers at floral development stage 12). Scale bars: A, B, 1 mm; C, D, 500 μm; E, 50 μm; F, 100 μm.

Our initial interest in miR159 was stimulated by its complementarity with a short region of transcripts encoding proteins of the GAMYB class (Rhoades et al., 2002; Park et al., 2002). The specific function of GAMYB proteins in various aspects of developmental regulation by the phytohormone GA led us to develop the hypothesis that miR159 might also function in GA-regulated developmental processes.

On the basis of the sequence complementarity between miR159 and transcripts encoding GAMYB proteins, it seemed possible that miR159 might direct the cleavage of these transcripts. We tested this possibility, and found that expression of miR159 in *N. benthamiana* causes a reduction in the level of intact *MYB33* transcripts and of detectable MYB33 protein. In addition, expression of miR159 failed to reduce the level of mutant *MYB33* transcripts (*mMYB33*) carrying an alteration in the miR159 complementary region. These results are consistent with the previous observation that *MYB33* and *MYB65* transcripts are cleaved in *Arabidopsis* plants in the centre of the miR159 binding site (Palatnik et al., 2003). Furthermore, whereas overexpression of *MYB33* has no obvious effect on development in *Arabidopsis*, overexpression of miR159-resistant *mMYB33* transcripts confers dramatic developmental perturbations [e.g. curly leaves (Palatnik et al., 2003)]. These results indicate that miR159-directed cleavage of *MYB33* (and other GAMYB-encoding transcripts) (Palatnik et al., 2003) is developmentally relevant. However, it is also possible that miR159 influences development via inhibition of translation of GAMYB-encoding transcripts.

We next investigated the levels of miR159 in *Arabidopsis* and barley GA-signalling mutant lines. We found that the accumulation of miR159 was positively regulated by GA, and negatively regulated by DELLA proteins (GAI and RGA in *Arabidopsis*; SLN in barley; Fig. 4). Therefore GA regulates miR159 accumulation via an evolutionarily conserved mechanism that involves opposition of DELLA function. Recent advances have shown that the DELLA proteins, although originally identified as components of the GA signalling pathway, also mediate auxin and ethylene responses (Achard et al., 2003; Fu and Harberd, 2003). Thus DELLA proteins may serve as a means of regulating miR159 levels in response to multiple hormonal signalling inputs. Although it has recently been shown that specific miRNA levels are hormonally regulated in *Drosophila* (Bashirullah et al., 2003), our results are, to our knowledge, the first demonstration of hormonal regulation of microRNA levels in plants.

Since GAMYB has been previously implicated in the regulation of flowering time in SDs in *Arabidopsis* (Blázquez et al., 1998; Blázquez and Weigel, 2000) and of anther development in barley and in rice (Murray et al., 2003; Kaneko et al., 2004), we determined if transgenic alteration in miR159 levels would perturb these developmental aspects. The timing of the floral transition is of considerable adaptive significance, and hence is subject to multiple interdependent genetic and environmental controls (Mouradov et al., 2002). Genetic and molecular analyses have identified three distinct genetic floral promotive pathways: the photoperiod pathway, the autonomous pathway and the GA pathway; the latter being of particular prominence in SD photoperiods (Simpson and Dean, 2003). Previous data suggested that the GA pathway promotes flowering in SD via GAMYB-dependent activation of the floral meristem identity gene *LEAFY* (Blázquez et al., 1998;

Blázquez and Weigel, 2000). Consistent with these ideas, we found that transgenic overexpression of miR159a resulted in a reduction in the levels of *MYB33* and of *LEAFY* transcripts, and a specific delay in flowering in SDs. Despite causing severe developmental perturbations at the vegetative rosette stage, expression of miR159-resistant *mMYB33* transcripts had no dramatic effect on flowering time in SDs (J. Palatnik and D. Weigel, personal communication). Similarly, treatment of SD-grown wild-type plants with GA promotes flowering time by only a few days (P.A. and N.P.H.; data not shown). Thus, in SD conditions, flowering time is at most slightly advanced by increases in MYB33 activity (due to *mMYB33* transcripts or GA treatment) but is markedly delayed by decreases in MYB33 activity (due to overexpression of miR159a).

The GA-deficient *ga1-3* mutant also either fails to flower or flowers very late in SD photoperiods (Wilson et al., 1992). Yet the *ga1-3* mutant contains reduced, rather than increased, levels of miR159 (Fig. 4A). Thus increased levels of mi159 cannot contribute to the late flowering of *ga1-3* in SD photoperiods. Our experiments further investigated the relationship between GA regulation of SD flowering and miR159 by looking at levels of transcripts encoding the floral integrator SOC1. GA regulates SD flowering time both via the GAMYB-*LEAFY* pathway, and via regulation of *SOC1* expression (Fig. 8) (Moon et al., 2003). We therefore investigated the effect of GA-DELLA signalling, and of overexpression of miR159a, on *SOC1* transcript levels. We showed that GA regulates *SOC1* transcript levels by opposing the negative effects of GAI and RGA (Fig. 4A). We also showed that transgenic overexpression of miR159a had no detectable effect on *SOC1* transcript levels (Fig. 5E). Thus GA regulates *SOC1* via a DELLA-opposition-dependent pathway that does not involve GAMYB (or miR159). It seems probable that SD-grown *ga1-3* plants flower late (although miR159 levels are reduced) because the reduction in *SOC1* transcripts, also seen in *ga1-3*, overcomes any promotion of flowering resulting from miR159 reductions.

We also found that transgenic overexpression of miR159a caused the formation of abnormal, sterile anthers. In particular, increased levels of miR159 caused an increase in anther size, a darkening of anthers and a failure to release pollen. These observations are of particular interest since, in barley, increased expression of HvGAMYB has some opposite effects on anther development. Whilst barley plants overexpressing *HvGAMYB* are also pollen-sterile, these plants have anthers of decreased size and lightened colour (Murray et al., 2003). In rice, lack of GAMYB also perturbs stamen and anther development (Kaneko et al., 2004). Taken together, these various observations suggest that a modulation in GAMYB levels causes a defect in anther development and a consequent reduction in floral fertility.

Overall, the results obtained from our transgenic overexpression experiments support the view that miR159 acts in GA signalling to regulate GAMYB activity, presumably through its ability to direct the cleavage of mRNA molecules encoding GAMYB proteins. However, our experiments also reveal a complexity in the relationship between GA signalling, miR159 and GAMYB levels. The levels of *MYB33* transcripts do not obviously differ between wild-type, GA-deficient (*ga1-3*) or GA-deficient plants lacking GAI and RGA (*ga1-3 ga-t6 rga-24*), despite the fact that miR159 levels vary between these different genotypes (Fig. 4A). At first sight this observation seems puzzling, since the *a priori* expectation would be that an increased level of miR159 should result in a reduction in *MYB33* transcript levels. However, the levels of another plant microRNA, miR39, actually display a positive correlation (rather than the expected negative correlation) with the levels of its cleavage target (Llave et al., 2002a). These unexpected relationships between microRNA and target transcript levels may result from a failure of gel-blot analyses to reveal underlying heterogeneities in the relative cell-specific distributions of microRNAs and their targets, or may reveal a homeostatic component of microRNA function.

Recent results indicate that miRNAs can work as components in negative feedback regulatory loops (Xie et al., 2003). It is therefore possible that miR159 acts within such a loop, as a general homeostatic regulator of GAMYB function. An illustrative example (Fig. 8) of how such feedback might operate could involve down-regulation of MYB33 activity by miR159 (as shown in this paper), and compensatory up-regulation of miR159 by MYB33. Indeed, potential GARE-like sites have been identified in the putative promoter of the miR159 precursor-encoding gene, suggesting the possibility of feedback regulation of miR159 by MYB33 (P.A. and N.P.H., data not shown).

Of course the above hypothetical example is but one possible route for regulation of levels of MYB33 activity, and concerns miR159 and MYB33 in isolation. The miR159/MYB33 system is also subject to DELLA-dependent regulation. In this case, the fact that *MYB33* transcript levels are indistinguishable in *ga1-3* compared with *ga1-3 gai-t6 rga-24* could be explained if absence of GAI and RGA increases both *MYB33* transcription and miR159 levels. Larger-scale changes to the system (e.g. transgenic overexpression of miR159a) break homeostatic regulation of *MYB33* levels and result in detectable changes in phenotype (delayed flowering in SD, perturbed anther development). We therefore propose that miR159 acts as a homeostatic regulator of GAMYB function in GA-regulated plant development. Despite these complexities, the strong evolutionary conservation of both sequence and phytohormonal regulation of miR159 suggests that this microRNA is of adaptive significance to the growth and development of many plant species.

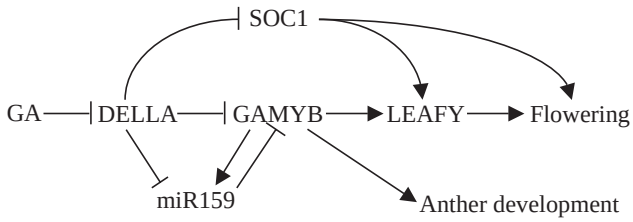

Fig. 8. The GA-DELLA signalling system regulates floral development by modulation of miR159/GAMYB and SOC1. Schematic representation of the regulation of the floral transition in SD photoperiods via the GA-DELLA signalling pathway. GA relieves the DELLA repression of GAMYB (e.g. MYB33) and SOC1 and enhances activity of the downstream floral meristem identity gene *LEAFY*. This activation is moderated by the GA activation of miR159, a post-transcriptional regulator of *GAMYB* transcript levels. This GA-dependent homeostatic mechanism provides sensitive regulation of the floral transition in SDs and anther development via the regulation of GAMYB levels.

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References

Achard, P., Vriezen, W. H., Van Der Straeten, D. and Harberd, N. P. (2003). Ethylene regulates Arabidopsis development via the modulation of DELLA protein growth repressor function. *Plant Cell* **15**, 2816-2825.

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

Bashirullah, A., Pasquinelli, A. E., Kiger, A. A., Perrimon, N., Ruvkun, G. and Thummel, C. S. (2003). Coordinate regulation of small temporal RNAs at the onset of *Drosophila* metamorphosis. *Dev. Biol.* **259**, 1-8.

Blázquez, M. A., Green, R., Nilsson, O., Sussman, M. R. and Weigel, D. (1998). Gibberellins promote flowering of *Arabidopsis* by activating the *LEAFY* promoter. *Plant Cell* **10**, 791-800.

Blázquez, M. A. and Weigel, D. (2000). Integration of floral inductive signals in *Arabidopsis*. *Nature* **404**, 889-892.

Cercos, M., Gomez-Cadenas, A. and Ho, T. H. D. (1999). Hormonal regulation of a cysteine proteinase gene, *EPB-1*, in barley aleurone layers: *cis*- and *trans*-acting elements involved in the coordinated gene expression regulated by gibberellins and abscisic-acid. *Plant J.* **19**, 107-118.

Chandler, P. M., Marion-Poll, A., Ellis, M. and Gubler, F. (2002). Mutants at the *Slender1* locus of barley cv Himalaya. Molecular and physiological characterization. *Plant Physiol.* **129**, 181-190.

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

Cheng, H., Qin, L., Lee, S., Fu, X., Richards, D. E., Cao, D., Luo, D., Harberd, N. P. and Peng, J. (2004). Gibberellin regulates *Arabidopsis* floral development via suppression of DELLA protein function. *Development* **131**, 1055-1064.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735-743.

Dill, A. and Sun, T.-p. (2001). Synergistic derepression of gibberellin signaling by removing RGA and GAI function in *Arabidopsis thaliana*. *Genetics* **159**, 777-785.

Emery, J. F., Floyd, S. K., Alvarez, J., Eshed, Y., Hawker, N. P., Izhaki, A., Baum, S. F. and Bowman, J. L. (2003). Radial patterning of *Arabidopsis* shoots by class III HD-ZIP and KANADI genes. *Curr. Biol.* **13**, 1768-1774.

Fu, X., Richards, D. E., Ait-ali, T., Hynes, L. W., Ougham, H., Peng, J. and Harberd, N. P. (2002). Gibberellin-mediated proteasome-dependent degradation of the barley DELLA protein SLN1 repressor. *Plant Cell* **14**, 3191-3200.

Fu, X. and Harberd, N. P. (2003). Auxin promotes *Arabidopsis* root growth by modulating gibberellin response. *Nature* **421**, 740-743.

Gocal, G. F. W., Sheldon, C. C., Gubler, F., Moritz, T., Bagnall, D. J., MacMillan, C. P., Li, S. F., Parish, R. W., Dennis, E. S., Weigel, D. and King, R. W. (2001). GAMYB-*like* genes, flowering, and gibberellin signalling in *Arabidopsis*. *Plant Physiol.* **127**, 1682-1693.

Gubler, F., Kalla, R., Roberts, J. K. and Jacobsen, J. V. (1995). Gibberellin-regulated expression of a *myb* gene in barley aleurone cells: evidence for Myb transactivation of a high-pI alpha-amylase gene promoter. *Plant Cell* **7**, 1879-1891.

Gubler, F., Raventos, D., Keys, M., Watts, R., Mundy, J. and Jacobsen, J. V. (1999). Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone. *Plant J.* **17**, 1-9.

Hamilton, A., Voinnet, O., Chappell, L. and Baulcombe, D. (2002). Two classes of short interfering RNA in RNA silencing. *EMBO J.* **17**, 4671-4679.

Harberd, N. P. (2003). Relieving DELLA restraint. *Science* **299**, 1853-1854.

Itoh, H., Ueguchi-Tanaka, M., Sato, Y., Ashikari, M. and Matsuoka, M. (2002). The gibberellin signaling pathway is regulated by the appearance and disappearance of SLENDER RICE1 in nuclei. *Plant Cell* **14**, 57-70.

Kaneko, M., Inukai, Y., Ueguchi-Tanaka, M., Itoh, H., Izawa, T., Kobayashi, Y., Hattori, T., Miyao, A., Hirochika, H., Ashikari, M. and Matsuoka, M. (2004). Loss-of-function mutations of the rice *GAMYB* gene impair α-amylase expression in aleurone and flower development. *Plant Cell* **16**, 33-44.

Kidner, C. A. and Martienssen, R. A. (2003). Macro effects of microRNAs in plants. *Trends Genet.* **19**, 13-16.

King, K. E., Moritz, T. and Harberd, N. P. (2001). Gibberellins are not required for normal stem growth in *Arabidopsis thaliana* in the absence of GAI and RGA. *Genetics* **159**, 767-776.

Koornneef, M. and van der Veen, J. H. (1980). Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. *Theor. Appl. Genet.* **58**, 257-263.

Koornneef, M., Elgersma, A., Hanhart, C. J., van Loenen-Martinet, E. P., van Rijn, L. and Zeevaart, J. A. D. (1985). A gibberellin insensitive mutant of *Arabidopsis thaliana*. *Physiol. Plant* **65**, 33-39.

Lee, H., Suh, S. S., Park, E., Cho, E., Ahn, J. H., Kim, S. G., Lee, J. S., Kwon, Y. M. and Lee, I. (2000). The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in *Arabidopsis*. *Genes Dev*. **14**, 2366-2376.

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

Llave, C., Kasschau, K. D., Rector, M. A. and Carrington, J. C. (2002b). Endogenous and silencing-associated small RNAs in plants. *Plant Cell* **14**, 1605-1619.

McGinnis, K. M., Thomas, S. G., Soule, J. D., Strader, L. C., Zale, J. M., Sun, T.-p. and Steber, C. M. (2003). The Arabidopsis *SLEEPY1* gene encodes a putative F-box subunit of an SCF E3 ubiquitin ligase. *Plant Cell* **15**, 1120-1130.

Moon, J., Suh, S. S., Lee, H., Choi, K. R., Hong, C. B., Paek, N. C., Kim, S. G. and Lee, I. (2003). The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in *Arabidopsis. Plant J.* **35**, 613-623.

Mouradov, A., Cremer, F. and Coupland G. (2002). Control of flowering time: Interacting pathways as a basis for diversity. *Plant Cell* **14**, S111-S130.

Murray, F., Kalla, R., Jacobsen, J. V. and Gubler, F. (2003). A role for HvGAMYB in anther development. *Plant J.* **33**, 481-491.

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

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

Peng, J., Carol, P., Richards, D. E., King, K. E., Cowling, R. J., Murphy, G. P. and Harberd, N. P. (1997). The *Arabidopsis GAI* gene defines a signalling pathway that negatively regulates gibberellin response. *Genes Dev.* **11**, 3194-3205.

Peng, J., Richards, D. E., Hartley, N. M., Murphy, G. P., Devos, K. M., Flintham, J. E., Beales, J., Fish, L. J., Worland, A. J., Pelica, F., Sudhakar, D., Christou, P., Snape, J. W., Gale, M. D. and Harberd, N. P. (1999). "Green revolution" genes encode mutant gibberellin response modulators. *Nature* **400**, 256-261.

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

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

Richards, D. E., King, K. E., Ait-ali, T. and Harberd, N. P. (2001). How gibberellin regulates plant growth and development: A molecular genetic analysis of gibberellin signalling. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 67-88.

Sasaki, A., Itoh, H., Gomi, K., Ueguchi-Tanaka, M., Ishiyama, K., Kobayashi, M., Jeong, D. H., An, G., Kitano, H., Ashikari, M. and Matsuoka, M. (2003). Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. *Science* **299**, 1896-1898.

Simpson, G. G. and Dean, C. (2002). Arabidopsis, the Rosetta stone of flowering time? *Science* **296**, 285-289.

Wilson, R. N., Heckman, J. W. and Sommerville, C. R. (1992). Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. *Plant Physiol.* **100**, 403-408.

Xie, Z., Kasschau, K. D. and Carrington, J. C. (2003). Negative feedback regulation of *Dicer-Like1* in *Arabidopsis* by microRNA-guided mRNA degradation. *Curr. Biol.* **13**, 784-789.

Yu, H., Xu, Y., Tan, E. L. and Kumar, P. P. (2002). AGAMOUS-LIKE 24, a dosage-dependent mediator of the flowering signals. *Proc. Natl. Acad. Sci. USA* **99**, 16336-16341.