GAMYB-like genes antagonise growth and promote PCD

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The miR159 regulated *GAMYB-like* genes inhibit growth and promote Programmed Cell Death in Arabidopsis

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Abstract

The microRNA159 family represses the conserved GAMYB-like genes that encode R2R3 MYB domain transcription factors that have been implicated in gibberellin (GA) signalling in anthers and germinating seeds. In Arabidopsis the two major miR159 family members, miR159a and miR159b are functionally specific for two GAMYB-like genes, MYB33 and MYB65. These transcription factors have been shown to be involved in anther development, but there are conflicting reports about their role in the promotion of flowering and little is known about their function in seed germination. To understand the function of this pathway, we identified the genes and processes controlled by these GAMYB-like genes. Firstly, we demonstrate that miR159 completely represses MYB33 and MYB65 in vegetative tissues. We show that GA does not release this repression and that these transcription factors are not required for flowering or growth. In contrast, the de-regulation of MYB33 and MYB65 in vegetative tissues in the absence of miR159 up-regulates genes that are GA-induced and highly expressed in the aleurone during seed germination. Confirming these genes are GAMYB-like regulated, their expression was dramatically reduced in myb33.myb65.myb101 seeds. Aleurone vacuolation, a GA-mediated programmed cell death (PCD) process required for germination, was impaired in these seeds. Finally, the de-regulation of MYB33 and MYB65 in vegetative tissues inhibits growth by reducing cell proliferation. Therefore, we conclude that miR159 acts as a molecular switch only permitting the expression of GAMYB-like genes in anthers and seeds. In seeds, these transcription factors participate in GA-induced pathways required for aleurone development and death.
INTRODUCTION

The GAMYB or GAMYB-like genes encode a highly conserved family of R2R3 MYB domain transcription factors that have been implicated in gibberellin (GA) signal transduction (Woodger et al., 2003). GAMYB was initially identified in the cereal aleurone, where its expression is induced by GA (Gubler et al., 1995). Here, it binds onto cis-acting GA-responsive elements leading to the transcriptional activation of genes encoding hydrolases required for starch mobilization during seed germination (Gubler et al., 1999). GAMYB is also strongly expressed in cereal anthers, especially in the tapetum, where it is also induced by GA (Murray et al., 2003; Aya et al., 2009). Emphasizing its importance, microarray analysis found that GAMYB is responsible for the majority of GA-regulated gene expression in both rice aleurone and anthers (Tsuji et al., 2006; Aya et al., 2009). In these tissues, GAMYB is involved in the Programmed Cell Death (PCD) of both the aleurone and tapetum, and in both tissues this process is GA-mediated (Guo and Ho, 2008; Aya et al., 2009). Conversely, GAMYB is negatively regulated by the microRNA (miRNA) family, miR159 (Tsuji et al., 2006). In rice, mature miR159 is present throughout the plant but is absent in the seed (Tsuji et al., 2006). In the anther, miR159 is co-expressed with GAMYB, and finely regulates the levels of this transcription factor (Tsuji et al., 2006).

In Arabidopsis there is a clade of seven closely related GAMYB-like genes that are potential targets of the three different MIR159 genes (Rhoades et al., 2002). Deep sequencing has found that miR159a and miR159b are overwhelmingly the predominant forms (Fahlgren et al., 2007) and using T-DNA loss-of-function mutants, these two MIR159 genes were demonstrated to be functionally redundant, since a mir159ab double mutant displayed pleiotropic developmental defects (Allen et al., 2007). Although all seven GAMYB-like genes contain potential miR159 binding sites in Arabidopsis, only MYB33 and MYB65 appeared de-regulated in mir159ab, a redundant gene pair that have similar expression patterns and functions (Millar and Gubler, 2005; Allen et al., 2007). The significance of this de-regulation was determined genetically, as all the developmental defects of mir159ab were suppressed in a mir159ab.myb33.myb65 quadruple mutant. This demonstrated that
the pleiotropic phenotype seen in \textit{mir159ab} is due solely to \textit{MYB33} and \textit{MYB65} activity. This was supported by expression of a miR159 resistant \textit{mMYB33} transgene (carrying a synonymous mutation of the miR159 binding site; Palatnik et al., 2003), that could phenocopy \textit{mir159ab} (Allen et al., 2007). Analysis of the reporter genes \textit{MYB33:GUS} and \textit{mMYB33:GUS} found that although \textit{MYB33} was transcribed broadly in the plant, miR159 appears to silence its expression everywhere but in seeds and anthers (Millar and Gubler, 2005). Therefore, similar to \textit{GAMYB} in cereals, \textit{MYB33} protein is predominantly expressed in anthers and seeds. In anthers, rice \textit{GAMYB} and \textit{MYB33}/\textit{MYB65} are likely to play a similar role. The rice \textit{gamyb} and \textit{myb33/myb65} mutants are male sterile due to the hypertrophy of the tapetum which expands to occupy the entire locule causing the microspores to degenerate (Millar and Gubler, 2005; Kaneko et al., 2004). In rice this was demonstrated to be caused by a failure of the tapetum to undergo PCD (Aya et al., 2009). In seeds the function of cereal \textit{GAMYB} and \textit{MYB33} and \textit{MYB65} may also be conserved, however no seed phenotype was apparent in \textit{myb33/myb65} (Millar and Gubler, 2005), although this may be due to further redundancy, as another close \textit{GAMYB-like} family member, \textit{MYB101}, is highly expressed in the seed (Penfield et al., 2006).

Unlike rice \textit{GAMYB} that is not involved in the transition to flowering (Kaneko et al., 2004), \textit{MYB33} and \textit{MYB65} have been implicated in the GA-signaling pathway regulating flowering under short-day conditions (Gocal et al., 2001; Achard et al., 2004). \textit{MYB33} mRNA levels were reported to increase in the shoot apex upon short to long-day shifts or GA-applications, treatments that also activate the expression of the flowering integrator gene \textit{LEAFY} (\textit{LFY}) and induce flowering (Gocal et al., 2001). As the \textit{LFY} promoter has a potential MYB binding site essential for its GA-activation (Blazquez and Weigel, 2000) to which \textit{MYB33} can bind, it was predicted that \textit{MYB33} may be the transcription factor transducing the GA-signal (Gocal et al., 2001). Supporting this hypothesis, the expression of a 35S:\textit{MIR159a} transgene in the Arabidopsis ecotype Lansberg \textit{erecta} decreased \textit{MYB33} and \textit{LFY} steady-state mRNA levels, and led to late-flowering under short-days (Achard et al., 2004). However, Schwab et al. (2005) also generated 35S:\textit{MIR159a} transgenic Arabidopsis lines [ecotype Columbia (Col)], but could find no alteration to \textit{MYB33} and \textit{MYB65}
transcript levels or flowering-time. Although a possible difference in ecotypes could be the explanation, further analysis is required to clarify the roles of MYB33 and miR159 in flowering.

Using physiological, microscopic and molecular analyses on myb33.myb65 and mir159ab mutants, we found that miR159 acts as a molecular switch confining the expression of MYB33 and MYB65 to seeds and anthers. This repression is not released by GA and consequently, MYB33 and MYB65 play no part in GA-mediated growth or flowering in vegetative tissues. We demonstrate that in seeds these GAMYB-like genes together with MYB101 regulate the expression of genes that are induced by GA during germination, and that they promote but are not essential for the progression of PCD in the aleurone. Therefore this study has redefined the biological role of the miR159-MYB33/MYB65 regulatory module.

RESULTS

**MYB33 and MYB65 are not essential for GA-mediated growth or flowering**

Previously, there have been conflicting reports on the involvement of MYB33 and miR159 in the promotion of GA-mediated flowering (Gocal et al., 2001; Achard et al., 2004, Schwab et al., 2005). In order to clarify the role of the miR159-MYB33/MYB65 pathway in GA-mediated floral induction, we examined the flowering-time and GA-response of the double mutants myb33.myb65 and mir159ab. Under long-day conditions the flowering-time of mir159ab was similar to wild-type (n = 34, P = 0.35), whereas myb33.myb65 flowered slightly earlier (n = 35, P = 4.71E-12; Fig. 1A). Under short-day conditions in which GA promotes flowering (Wilson et al., 1992), myb33.myb65 had the same flowering-time as wild-type (n= 30 and 31 respectively, P = 0.52; Fig. 1B). Conversely, mir159ab flowered later and only 23 out of 32 plants flowered after 110 days after sowing. The flowering-time of the 23 plants was delayed twenty days (P < 10^-5; Fig. 1B). As mir159ab has a strong morphological phenotype, it is unsure whether this delay is due to the morphological defects of mir159ab or to MYB33 and MYB65 expression per se. However it is clear that MYB33 and MYB65 de-regulation in mir159ab does not promote flowering.
To examine the GA-response of myb33.myb65 and mir159ab, we applied a series of GA treatments and scored their flowering-time under short-day conditions. The response of myb33.myb65 was similar to wild-type, since plants sprayed with GA flowered approximately 20 days before the control plants (Fig. 2A). However the effect of GA on mir159ab plants was more subtle. Although the flowering-time of GA-treated mir159ab was substantially later than GA-treated myb33.myb65 and wild-type, all GA-sprayed mir159ab plants flowered (n=16), whereas only 7 out of 12 ethanol-sprayed mir159ab plants flowered after 110 days. These data demonstrate that MYB33 and MYB65 are not the main effectors mediating the flowering response to GA.

We measured the effect of GA-application on the steady-state transcript levels of MYB33, MYB65 and the levels of mature miR159a and miR159b in the wild-type shoot apex regions (SARs). We also measured the transcript levels of the GA-responsive genes LEAFY (LFY), GIBBERELLIN 3 BETA-HYDROXYLASE 1 (GA3OX1; Cowling et al., 1998) and SCARECROW-LIKE 3 (SCL3; Ogawa et al., 2003) in myb33.myb65 and wild-type SARs to confirm that the plants were responding to the GA treatment. The levels of MYB33, MYB65, miR159a and miR159b remained unchanged after the application of GA (Fig. 2B). Conversely, the transcript levels of LFY were slightly up-regulated and the levels of GA3OX1 and SCL3 were down-regulated in GA-treated myb33.myb65 and wild-type (Fig. 2C). These controls confirmed that the plants had perceived and responded to the hormone.

We also analysed the requirement of MYB33 and MYB65 for GA-mediated growth of long-day grown rosettes. To quantify this, we measured the petiole length of third leaves, as this has been shown to be a good indicator of GA-response in the ecotype Columbia (Gocal et al., 2001). Again, myb33.myb65 responded to GA similar to wild-type plants (n= 25; Fig. 2D), and MYB33 and MYB65 mRNA levels failed to change with GA application (Fig. 2E). In contrast, mir159ab did not display a growth response upon GA-application (n= 25; Fig. 2D). Taken together, these data demonstrate that MYB33 and MYB65 are not required for flowering under short-day conditions or for GA-induced growth and that their mRNA levels are not up-regulated
by GA in the Arabidopsis ecotype Columbia. Finally, no dramatic induction of *MYB33* or *MYB65* was detected in SARs in the Arabidopsis ecotype Landsberg *erecta* (Fig S1), implying that the mRNA levels of the *GAMYB-like* genes in SARs are independent of GA in both ecotypes.

**MiR159 represses *MYB33* expression to biologically inconsequential levels in vegetative tissues**

Since *myb33.myb65* displayed a wild-type phenotype in all the physiological aspects tested, this raised the issue of whether *MYB33* and *MYB65* have any role in vegetative tissues. To determine this, we firstly analysed the expression of a *MYB33:GUS* transgene in the SAR. We had previously shown that miR159 represses *MYB33* in these tissues (Millar and Gubler, 2005). However, as *MYB33* mRNA is still detectable at the SAR (Allen et al., 2007), we wanted to ascertain whether MYB33 protein is absent or whether it is restricted to a select group of cells by miR159. Of five independent *MYB33:GUS* lines that had previously shown strong staining in anthers (Millar and Gubler, 2005), very weak staining was only observable in three lines after extended staining periods (five days) and tissue clearing (Fig. 3A-B). In these three lines, GUS staining appeared present in all cells of the SAR (Fig. 3A). By contrast, the expression of the *mMYB33:GUS* transgene (miR159-resistant; Millar and Gubler, 2005) in five independent lines was extremely strong throughout the SAR and staining appeared after only 16 hours of incubation with the substrate (Fig. 3C). Taken together, these results suggest that *MYB33* expression is strongly repressed by miR159 throughout the SAR. This applies to other vegetative tissues, since no GUS signal was detected in leaves or roots of *MYB33:GUS* plants even after prolonged staining.

To further characterize the silencing of *MYB33* by miR159, we measured both mRNA levels and GUS activity of the transgenes in five independent *MYB33:GUS* and *mMYB33:GUS* lines, where we assumed that GUS activity reflects MYB33:GUS protein levels. On average, *mMYB33:GUS* mRNA levels were approximately ten-fold higher than those of *MYB33:GUS* (Fig. 3E). In contrast, GUS activity levels were on average almost 400-fold higher in *mMYB33:GUS* compared to *MYB33:GUS* lines.
As GUS activity in MYB33:GUS lines (4.64 ± 2.83) was only slightly higher than in non-transgenic lines (normalized to zero), such a high fold-level difference appears due to the almost complete absence of MYB33:GUS protein. Together, these data suggest that miR159 represses MYB33 expression not only by reducing mRNA levels, but also by repressing the translation of any remaining MYB33 mRNA.

Finally, we carried out a microarray analysis on the SAR of 15-day-old myb33.myb65 plants. Very few genes were found to be differentially expressed between wild-type and myb33.myb65. Only two genes met the P ≤ 0.005, 2-fold change criteria [invertase (At1g62770), fold-change = 2.19, P < 0.003; and Expressed protein (At3g52060), fold-change = 2.14, P < 0.003]. Relaxation of the p-value (P < 0.05), but with a three-fold change cut-off again found only two genes to be differentially expressed [formaldehyde dehydrogenase (At5g14780), fold-change = 3.2, P < 0.006; and nitrate reductase 1 (At1g77760), fold-change = -3.84, P < 0.006]. Thus, the transcriptomes of wild-type and myb33.myb65 SAR are almost identical, which supports the notion that miR159 completely represses these transcription factors under normal conditions in the SAR.

MYB33 and MYB65 activate the expression of aleurone-related genes in mir159ab vegetative tissues

Next, we performed a transcriptomic analysis on the SAR of 15-day-old mir159ab plants in order to identify genes regulated by MYB33 and MYB65 and elucidate their biological role. Using a two-fold change and P < 0.005 cut off, we found 121 up-regulated and 45 down-regulated genes in mir159ab when compared to wild-type (for a complete list see Table S1). To validate that these genes were differentially expressed, qRT-PCR was performed on thirty six of these genes that covered a broad range of fold-change levels. In all instances, gene expression was confirmed to be altered, and in many cases the fold-changes determined by qRT-PCR were similar to those determined by the microarrays, implying that the microarray data were highly reliable (Table 1, S1). As expected, MYB33 and MYB65 were among the up-regulated genes in mir159ab as they are no longer repressed by miR159 (Allen et al., 2007).

Only one other predicted miR159 target was found to be
up-regulated, OLIGOPEPTIDE TRANSPORTER1 (OPT1; Table S1), and no low complementary targets were up-regulated (up to seven mis-matches; http://bioinfo3.noble.org/miRNA/miRU.htm). This, the first report of a microarray performed on a loss-of-function miRNA mutant, is consistent with the notion that plant miRNAs have highly specific effects on the transcriptome (Schwab et al., 2005). This also confirms that the majority of gene expression changes in mir159ab are due to MYB33 and MYB65 de-regulation. In agreement with this, the analysis of gene expression in the rosettes of a mMYB33 transgenic line that had a phenotype indistinguishable from mir159ab plants (Line 2; Allen et al., 2007) revealed similar gene expression changes to mir159ab (Table 1).

Interestingly, 16 out of the 39 genes that are more than 3-fold up-regulated in mir159ab are induced by GA in 3, 6 or 9 hour-imbibed seeds of the GA-deficient mutant ga1-3 according to microarray data (Fig. 4A; Schmid et al., 2005). To confirm this, we carried out qRT-PCR analysis on 14 of the mir159ab up-regulated genes and found eight of them to be induced by GA in 30 hour-imbibed ga1-3 seeds (Fig. 4B). This analysis identified three more genes that are up-regulated by GA in the seed: BETAXYLOSIDASE1 (BXL1; At5g49360), GA-REGULATED GENE1 (GASA1; At1g75750) and a putative cysteine proteinase (CP; At3g45310). Therefore approximately half of the genes identified on the arrays as being more than 3-fold up-regulated in mir159ab shoot apex tissues are induced by GA in the seed. Of these genes, CYSTEINE PROTEINASE1 (CP1; At4g36880), GASA1 and DISRUPTED MEIOTIC CONTROL1 (DMC1; At3g22880), three of the most up-regulated genes in our array, had been previously shown to be induced by GA in the seed (Ogawa et al., 2003; Bouquin et al., 2001).

According to microarray data, many of these seed GA-responsive genes are predominantly expressed in the aleurone, where MYB33 and MYB65 are also preferentially transcribed (Fig. 4C; Penfield et al., 2006, Winter et al., 2007). Moreover, nine of these genes had an aleurone microarray expression value of over 1000, indicating that they are very strongly expressed in this tissue (Fig. 4C; Penfield et al., 2006, Winter et al., 2007). In addition, many of the up-regulated genes encoded transporters and hydrolases (Table S2). Most of the transporters up-
regulated in the array are predicted to transport nutrients, like sugars, amino-acids and proteins. Among the up-regulated hydrolases, seven were predicted to encode proteases and five are predicted or have been confirmed to be involved in cell wall degradation (Table S2). Functions such as this would be consistent with the secretory role of the aleurone and its final progression to Programmed Cell Death (PCD) through autophagy (Fath et al., 2000). All these data strongly supports the notion that the GAMYB class of transcription factors are positive regulators of GA-signalling during seed germination (Gubler et al., 1995; Woodger et al., 2003). Finally, the function of these GAMYB transcription factors is highly conserved, as many common homologues are up-regulated by GAMYB in \textit{mir159ab} vegetative tissues and in anthers and embryoless half seeds of rice (Table 1; Tsuji et al., 2006).

As \textit{MYB33} and \textit{MYB65} are globally de-regulated in \textit{mir159ab} (Allen et al., 2007), we examined whether these aleurone-related genes have been also up-regulated throughout \textit{mir159ab}. qRT-PCR found that \textit{CP1}, \textit{GASA1}, \textit{DMC1}, \textit{BLX1} and \textit{BXL2} genes were all up-regulated in whole rosette and floral tissues of \textit{mir159ab} (Table 1). The fact that \textit{CP1}, \textit{BLX1} and \textit{BLX2} were not induced in \textit{mir159ab} seeds probably reflects that they are already highly expressed in this tissue. However, in addition to these aleurone-related genes, many other genes were found to be globally affected (Table 1, S1). This includes \textit{ALLENE OXIDASE SYNTHASE1} (\textit{AOS1}) and \textit{ACC OXIDASE 1} which code for key enzymes in jasmonic acid and ethylene biosynthesis respectively. This suggests that the overall gene expression changes observed in \textit{mir159ab} likely result from alteration of numerous pathways.

The up-regulated \textit{mir159ab} genes are down-regulated in \textit{myb33.myb65.myb101} seeds

The up-regulation in \textit{mir159ab} of many genes highly expressed in the aleurone, prompted us to examine whether \textit{MYB33} protein is in fact present in this tissue. To examine this, we stained and sectioned 30 h imbibed \textit{MYB33:GUS} seeds. \textit{MYB33} expression was detected in the embryo (Fig. 5A) and was especially strong throughout the aleurone layer (Fig. 5B), confirming that the presence of GAMYB
activity in the aleurone is conserved in both monocotyledonous and dicotyledonous plants. If the genes up-regulated in mir159ab SAR are activated by MYB33 and MYB65 during germination, then their expression should be down-regulated in myb33.myb65 seeds. We measured the mRNA abundance of CP1, CP, GASA1, BXL1 and BXL2 in 30 h imbibed myb33.myb65 seeds. We chose those genes because they were up-regulated by GA in 30 h imbibed ga1-3 seeds (Fig. 4B). All these genes were slightly down-regulated in myb33.myb65 with the exception of CP1 (Fig. 5C). However further redundancy of GAMYB activity in seeds is likely, as MYB101, another GAMYB-like gene, is highly transcribed in the aleurone (Penfield et al., 2006). We obtained a myb101 mutant from the SALK collection (SALK_061355) in which the T-DNA had inserted within the second exon of the gene (Fig. 5D). qRT-PCR analysis determined that the expression of the myb101 allele was 100 fold lower compared to MYB101 allele (Fig. 5E). Despite this, the myb101 mutant displayed a wild-type phenotype. In myb101 seeds the levels of GASA1, BXL1 and BXL2 were reduced to the same extent as in myb33.myb65 seeds (Fig. 5C). Using this myb101 allele we obtained the myb33.myb65.myb101 triple mutant that displayed a wild-type phenotype except for male sterility similar to myb33.myb65. The mRNA levels of CP1, CP, GASA1, BXL1 and BXL2 were reduced two fold or more in myb33.myb65.myb101 seeds (Fig. 5C). These data demonstrate that these three transcription factors are regulating similar genes in seeds, and that there exists a tight regulatory relationship between GAMYB-like activity and the mRNA abundance of these five genes, whether it is in the aleurone of wild-type plants or in vegetative tissues of mir159ab.

As CP1, CP, GASA1, BXL1 and BXL2 are GA-regulated in the seed, we investigated whether any of the three GAMYB-like genes could be candidates for transducing this GA signal in seeds by measuring their mRNA levels in GA treated ga1-3 seeds. Only MYB101 mRNA levels were increased upon GA treatment (Fig.5F-H). Therefore although MYB33 and MYB65 are controlling the expression of seed GA-regulated genes, their mRNA abundance does not appear GA-regulated in the seed.

**MYB33, MYB65 and MYB101 promote PCD in the aleurone**
As GAMYB has been implicated in the PCD of the aleurone in barley (Guo and Ho, 2008), we examined whether this process is compromised in \textit{myb33.myb65.myb101}. We visualized the aleurone layers of \textit{myb33.myb65.myb101} under UV light which makes Protein Storage Vacuoles (PSV) to fluoresce (Bethke et al., 2007). Shortly after imbibition aleurone cells contain many PSV but during germination they coalesce resulting in a decrease in their numbers (Bethke et al., 2007). At late stages of germination only one big lytic vacuole occupies the aleurone cell. This process is called vacuolation. We determined the vacuolation rate of the \textit{myb33.myb65.myb101} aleurone layers. We distinguished between the area of the aleurone that is in contact with the embryo shoot and the area of the aleurone in contact with the radicle as they have different vacuolation rates (Bethke et al., 2007). The vacuolation rate of the area of the aleurone in contact with the radicle was slower in \textit{myb33.myb65.myb101} compared to the wild-type, as 24 h and 30 h after imbibition the mutant aleurone cells contained more PSV (P < 0.0001; Fig. 6A). However, the triple mutant was able to vacuolate completely after 48h. No difference was seen in the aleurone in contact to the embryo shoot.

It has been previously shown that isolated aleurone layers that are incubated at 30°C do not vacuolate (Fig. 6B) unless they are supplemented with GA (Fig. 6C; Bethke et al., 2007). To determine if the Arabidopsis \textit{GAMYB-like} genes are involved in this GA process we incubated \textit{myb33.myb65.myb101} and wild-type aleurone layers at 30°C with and without GA and counted the number of aleurone layers that had vacuolated after five days. When incubated in the control media, 50% of the wild-type and mutant aleurone layers had vacuolated after five days (Fig. 6D). However, only 70% of the mutant aleurone layers incubated with GA vacuolated compared to 100% of the wild-type aleurone layers (Fig. 6D). This data demonstrates that \textit{MYB33}, \textit{MYB65} and \textit{MYB101} promote, but are not essential for the vacuolation of the aleurone. Accordingly, the triple mutant seeds germinate as efficiently as wild-type ones under normal conditions (data not shown).

\textit{MYB33} and \textit{MYB65} antagonise growth in vegetative tissues through inhibition of cell proliferation
As the up-regulated genes in mir159ab might be involved in the progression of PCD in the aleurone, we wanted to determine if cells in the aerial organs of mir159ab were undergoing PCD as a result. However staining of 13 day-old rosettes with the dye trypan blue, that stains dead and dying cells failed to find any evidence of enhanced PCD in mir159ab rosettes (Fig. 7). Therefore we carried out a microscopic analysis of mir159ab to determine the consequences of the up-regulation of MYB33 and MYB65 in mir159ab.

Anatomical differences appeared very early on mir159ab development. The shoot apical meristem (SAM) of four-day-old mir159ab plants appeared dome shaped and enlarged, compared to the flat shoot apical meristem of wild-type plants (n=5; Fig. 8A-B). An enlarged SAM was also present in 14-day-old (n=2; Fig. 8C-D) mir159ab plants. The organization of the tissue layers of the mir159ab SAM appeared normal, as the L1, L2 and L3 layers were clearly distinguishable, although the mir159ab SAM had more cells in L3 and subtending meristematic (non-vacuolated) tissue (Fig. 8D). Newly initiated mir159ab rosette leaves are flat, but gradually curl upwards with time (Allen et al., 2007). We studied the structure of 24-day-old fifth leaves of mir159ab that were curled at that age. They were either transversely sectioned (Fig. 9A-B) or cryo-fractured and analysed with SEM (Fig. 9C-D). The most notable difference in mir159ab leaves was that mesophyll cells were considerably larger than their wild-type counterparts (Fig. 9A-B). Cell size measurements determined that mir159ab palisade and spongy mesophyll cells were approximately 217% and 274% larger, respectively (Fig. 9E). mir159ab leaves also had approximately 58% fewer mesophyll cells per mm² than wild-type leaves (Fig. 9F). Since mir159ab leaves are also smaller, this implies that they contain far fewer mesophyll cells.

Further analysis was performed using SEM on the epidermal surfaces of mir159ab and wild-type leaves (Fig. 9G-J). Similar to the mir159ab mesophyll layers, both abaxial and adaxial surfaces of mir159ab had fewer cells when compared to wild-type (Fig. 9K). However, the mir159ab epidermal cells were smaller (Fig. 9L). This reduction in cell number and size was more pronounced on the adaxial surface, causing the ratio between the lengths of the adaxial and abaxial surfaces to be
reduced from $0.98 \pm 0.02$ (n=2) in the wild-type to $0.79 \pm 0.02$ (n=2) in mir159ab leaves. This may have resulted in the upward curling of the leaves. Furthermore, the number of cells composing the vascular bundles was considerably reduced in both xylem and phloem (Fig. 9M-N) and the venation pattern of cotyledons and leaves was also simpler (Fig. 9O-R). This, together with the fact that all organs of mir159ab plants are smaller (Allen et al., 2007), suggests that the major consequence of the de-regulation of MYB33 and MYB65 in mir159ab is a reduction of cell proliferation.

We reasoned that this disruption of cell proliferation could be due to the up-regulation of cell cycle inhibitor genes. We measured the expression levels of the seven members of the KIP-RELATED PROTEIN (KRP; De Veylder et al., 2001) family of cell cycle inhibitors and found that KRP7 was up-regulated throughout mir159ab (Fig. S2). However an RNAi construct generated to silence KRP7 in the mir159ab background failed to suppress the mir159ab phenotype (Fig. S2). This suggested that the increased KRP7 levels in mir159ab are only indicative of the decrease in cell proliferation. However it is clear that expression of MYB33 and MYB65 in vegetative tissues antagonises growth through the disruption of cell proliferation, an outcome completely counter-intuitive to the notion that these GAMYB-like genes promote GA-mediated growth.

DISCUSSION

Although much is known about plant miRNAs and their regulatory relationship with their target genes, what downstream genes or processes these regulatory modules control is in most instances unknown. Here we have identified a set of genes activated by MYB33 and MYB65 that are usually strongly expressed in the aleurone, but are globally up-regulated in mir159ab. GAMYB-like activity in the aleurone promotes PCD, whereas in rosette tissues it antagonises cell proliferation (Fig 10). Although these activated genes are GA up-regulated, this is restricted to seeds, as MYB33/MYB65 activity appears fully suppressed in vegetative tissues by miR159, where they are not involved in GA-mediated growth or flowering. Based on
these findings, miR159 could be regarded as a molecular switch that confines
MYB33 and MYB65 to seeds and anthers where we have shown here that they
promote PCD in the aleurone, and it is likely that they also do in the tapetum.

miR159a and miR159b act as “switch miRNAs’ in vegetative tissues

We have three lines of evidence supporting the notion that despite MYB33
and MYB65 being consistently transcribed throughout Arabidopsis, their protein
levels are suppressed to biologically insignificant levels except in seeds and anthers.
Firstly, the very weak expression of MYB33:GUS contrasts to the intense expression
of the mMYB33:GUS transgene in vegetative tissues, implying that although MYB33
is being transcribed, very little MYB33 protein accumulates. This silencing appears
not only due to the reduction of MYB33 mRNA levels, but also the inhibition of the
translation of the remaining MYB33 transcript, as highlighted by the discrepancies
between mRNA and GUS activity levels of the MYB33:GUS transgene (Fig. 3E-F).
MiRNA-mediated translational repression mechanisms appear common in plants
(Brodersen et al., 2008), which in this case may fully ensure the complete silencing
of MYB33. This complete silencing is supported by the wild-type phenotype of
myb33.myb65 at the vegetative stage and the negligible differences between the
SAR transcriptomes of wild-type and myb33.myb65. In this respect, miR159 could be
regarded as a switch miRNA (Bartel and Chen, 2004), fully repressing MYB33 and
MYB65. As miR159a and miR159b are redundant, they must be made in large
excess to carry out this repression, and this is especially so considering that
miR159a is 10-fold more abundant than miR159b (Rajagopalan et al., 2006;
Fahlgren et al., 2007) but a mir159a mutant still appears phenotypically
indistinguishable from wild-type (Allen et al., 2007). Thus, increasing miR159 levels
even further would be predicted to have little effect and this is what Schwab et al.
(2005) found when they overexpressed miR159a, as 35S:MIR159a transgenic plants
did not exhibit any aberrant phenotype apart from male sterility and had unaltered
mRNA levels of MYB33 and MYB65.

MYB33 and MYB65 are not essential in the GA-response for vegetative growth
or flowering
Previous reports found MYB33 mRNA levels up-regulated by GA and associated with the induction of LFY and flowering under short-days (Achard et al., 2004). However, our work shows that MYB33 and MYB65 are not essential for flowering under short-day conditions, as the myb33.myb65 double mutant has a wild-type flowering time and responds to GA treatments to the same extent as wild-type plants (Fig. 1 and 2). It could be argued that in the absence of these transcription factors, the other members of the GAMYB-like family take over their role in the flowering pathway. However this seems highly improbable, as transcription of these members is restricted to anthers and seeds (Zimmerman et al., 2004) and they were not up-regulated in the SAR of myb33.myb65 (data not shown). Furthermore, no flowering-time related genes were up-regulated in mir159ab according to the microarray analysis, consistent with the fact that mir159ab exhibits a delayed flowering-time, going against the view that MYB33 and MYB65 are activators of flowering. Finally, we failed to detect any GA-induction of MYB33 or MYB65 mRNA in vegetative tissues upon GA treatment despite the plants displaying clear physiological GA-responses. Furthermore, as the levels of miR159 did not change with GA application, it is unlikely that MYB33 and MYB65 protein levels increase due to the release of the translational repression by miR159. Therefore, our data suggests that MYB33 and MYB65 do not transduce the GA-signal in vegetative tissues of Arabidopsis.

The GAMYB-like genes promote GA-mediated PCD in the aleurone

However, we have linked MYB33, MYB65 and MYB101 to a GA-mediated response in the aleurone. This process appears conserved between monocotyledonous and dicotyledonous plants, but has been best characterised in cereals. Here the embryo produces GA during germination that stimulates the aleurone and transforms it into a secretory tissue that synthesizes a spectrum of hydrolases for the mobilization of nutrients in the endosperm. These hydrolases are synthesized de novo from amino acids that arise from the breakdown of the proteins stored in the Protein Storage Vacuoles (PSV) that are numerous in mature aleurone cells. As a consequence of the hydrolysis, these organelles swell and coalesce to form a big lytic vacuole that at the end of germination collapses and results in cell
death (reviewed in Fath et al., 2000). GAMYB in cereals activates the expression of hydrolytic enzymes in response to GA (Gubler et al., 1999) and is also involved in the progression of PCD (Guo and Ho, 2008). Although Arabidopsis aleurone has been less characterized, it is known that also undergoes GA-mediated vacuolation (Bethke et al., 2007) as the cellular sources of nutrients are catabolised and exported to the growing seedling. Our work shows that MYB33, MYB65 and MYB101 are involved in these GA-regulated aleurone processes. We have demonstrated that the vacuolation of the myb33.myb65.myb101 aleurone is impaired when compared to wild-type (Fig. 6). Interestingly, there is a differential response between the aleurone cells surrounding the zone at which the root penetrates the endosperm, to aleurone cells on the shoot side. As the aleurone layer and the penetration of the radicle through the endosperm are factors controlling seed dormancy (Bethke et al. 2007), the GAMYB-like genes could be involved in controlling this important seed trait. In addition to this slower rate of vacuolation, there is a reduction in the expression of genes that would be predicted to be involved in this process. Firstly, the expression of two highly expressed cysteine proteinases was reduced in myb33.myb65.myb101 germinating seeds. Proteases are associated with the mobilization of storage proteins early during germination but at later stages they are correlated with autolysis and cell death (reviewed in Fath et al., 2000). We speculate that CP1 and CP may be important cysteine proteinases carrying out such functions. Supporting this, the rice ortholog of CP1 has been related to PCD in the anther. The oscp1 loss-of-function mutant is male sterile (Lee et al., 2004) and Li et al. (2006) showed that the expression of CP1 is up-regulated by the transcription factor TAPETUM DEGENERATION RETARDATION (TDR) that is required for the PCD of the tapetum to occur. CP1 is most likely a direct target of TDR, as this transcription factor is able to bind to the CP1 promoter (Li et al., 2006). Secondly, the closely related genes BXL1 and BXL2 are similarly reduced in myb33.myb65.myb101. They encode beta-xylosidases that are predicted to be targeted to the extracellular matrix (Goujon et al., 2003). BXL1 has been characterised further and has been found to have α-L-Arabinofuranosidase and β-D-Xylosidase activity (Minic et al., 2004), so it can hydrolase xylans, the major component of the hemicelluloses of the cell wall. It has been shown to be induced by sugar starvation, possibly to release sugars from the cell wall and provide a source of carbon (Lee et al., 2007). Moreover there is evidence that BXL1 is required for weakening the outer primary cell of the seed coat
to allow the release of the mucilage upon seed imbibition (Arsovski et al., 2009). Therefore, we propose that \textit{BXL1} and \textit{BXL2} may participate in an analogous role in the aleurone, degrading and weakening the aleurone cell wall, as during imbibition aleurone cells that are located near the radicle become spherical, indicating the cell walls are thinning and weakening (Bethke et al., 2007), a process that may precede the degradation of the aleurone cell wall to allow radicle penetrance. All four of these genes (\textit{CP1}, \textit{CP}, \textit{BXL1} and \textit{BXL2}) plus many other hydrolases and transporters identified as being regulated by \textit{MYB33} and \textit{MYB65} are GA-regulated in the seed, giving further credence that these genes are part of the aleurone response to GA. Moreover the mRNA levels of \textit{GASA1}, \textit{BXL1}, \textit{BXL2} and \textit{CP} correlate tightly with GAMYB-like activity, with intermediate reductions in \textit{myb33.myb65} and \textit{myb101}, but even further reductions in \textit{myb33.myb65.myb101}. This demonstrated that these three GAMYB-like genes are rate-limiting in terms of the expression of these genes, however they are redundant with regards to the PCD of the aleurone, as no changes in vacuolation rates were seen in \textit{myb33.myb65} or \textit{myb101} mutants (data not shown). Interestingly, only \textit{MYB101} mRNA levels were found to be up-regulated by GA in the seeds (Fig. 5). \textit{MYB65} and \textit{MYB33} might be regulated by GA post-transcriptionally or alternatively not be regulated by GA at all. However, the fact that only the triple mutant displayed a slower vacuolation rate together with an altered response to GA (Fig.6) suggests that all three transcription factors are involved in the transduction of the GA signal. In conclusion, we propose that we have not only identified the GAMYB-like transcription factors involved in these GA-mediated aleurone pathways, but also likely many genes that encode hydrolases and transporters that may be the effectors of the processes of secretion and PCD in the aleurone (Fig. 10). However to prove the latter it is necessary to determine whether the GA-induction of these aleurone-related genes is attenuated in a \textit{myb33.myb65.myb101.ga1-3} quadruple mutant and demonstrate that vacuolation is impaired in mutants of these aleurone-related genes.

**Analogous gene functions in the aleurone and tapetal cell layers?**

As in cereals, Arabidopsis GAMYB-like expression is strong in the aleurone and tapetum (Fig. 5; Millar and Gubler, 2005). These tissues share analogous
biological functions, providing rapidly dividing organs (the embryo and pollen grains) with nutrients and undergoing PCD in the process (reviewed in Rogers, 2005). In myb33.myb65 the tapetum fails to degenerate (Millar and Gubler, 2005) and also aleurone PCD is compromised in myb33.myb65.myb101. Thus, it is likely that a subset of the genes identified in this study are activated by MYB33 and MYB65 in the anther or even in both aleurone and anther. For instance, DMC1 and CP1 were found to be induced by GA in the seed, but DMC1 is also involved in the progression of meiosis (Couteau et al., 1999) and CP1 is necessary for male fertility in rice (Lee et al., 2004). Interestingly, microarray analysis in rice found that GAMYB activates different sets of genes in anthers and seeds (Tsuji et al., 2006), which appears at odds with the scenario in Arabidopsis, as many of the aleurone-related genes were globally up-regulated in mir159ab, including flowers (Table 1). However it is unknown whether these aleurone-related genes are indeed the most MYB33/MYB65 up-regulated set of genes in Arabidopsis anthers, especially considering that the most up-regulated gene in mir159ab flowers we found was only five-fold higher. Furthermore, although PCD is common to both the aleurone and tapetum, it is likely that MYB33 and MYB65 are performing many specialized roles in the anther that do not occur in the aleurone. Therefore microarray analysis of wild-type versus myb33.myb65 anthers would be needed to resolve this question. Moreover the anther is a tissue where MYB101 is strongly transcribed (Allen et al., 2007) adding further complexity. Unlike the seed, myb33.myb65 anthers display a mutant phenotype, suggesting sub-functionalization of MYB101 with regard to MYB33 and MYB65. As a myb101 mutant is not male sterile, a detailed comparison of myb33.myb65 with myb33.myb65.myb101 may be needed to uncover any role MYB101 has in the anther.

**MYB33 and MYB65 inhibit cell proliferation in vegetative tissues**

It is clear from our transcript profiling analysis that many genes that are normally transcribed strongly in the aleurone have been globally up-regulated in mir159ab. Despite this occurring, we can find no evidence for PCD taking place in mir159ab rosette tissues. Instead, reduced cell proliferation occurs in mir159ab as indicated by our anatomical analysis of mir159ab leaves that exhibit fewer cells in all
the tissues and also larger cells in the mesophyll. This larger cell volume in association with reduced cell numbers is indicative of the phenomenon called “compensation” that consists in the increase of the volume of each cell triggered by a reduction in cell proliferation in order to maintain a wild-type leaf size (Tsukaya, 2008). Reduced cell proliferation could be a secondary effect to the activation of PCD processes that usually occur in the aleurone. In animal systems the coordination of PCD and cell proliferation has been comprehensively demonstrated, where arrest or disruption of the cell cycle is a common feature of cells that eventually undergo PCD (Gilchrist, 1998). Furthermore, it has been shown that many PCD factors such as p53 are able to trigger cell cycle arrest and apoptosis (Aylon and Oren, 2007). According to our hypothesis, the activation of MYB33/MYB65 in mir159ab vegetative tissues is not sufficient to lead to death as we showed by staining rosette leaves with trypan blue (Fig. 7), but does lead to a reduction in cell numbers.

MATERIALS AND METHODS

Plant material, growing conditions and physiological analysis

All Arabidopsis thaliana seeds were sterilized and stratified at 4°C in the dark, and then grown in 22°C growth cabinets under fluorescent illumination of 130-150 µmol·m⁻²·s⁻¹ on long-day (16 h of light) or short-day (10 h of light) photoperiods in metro-mix soil. The double mutants myb33.myb65 and mir159ab have been previously described (Millar and Gubler, 2005; Allen et al., 2007). myb33.myb65 was in a mixed Columbia-6 (myb33) and Columbia-0 (myb65-2) background and ga1-3 was in a Columbia-0 background.

For determination of flowering-time, we scored days to flowering when flowers were visible in the shoot apex by naked eye. For the GA-induction of flowering under short-day conditions, 22 day-old plants were sprayed twice weekly for three weeks with 20% ethanol or 100 µM GA₄ dissolved in 20% ethanol and their flowering-time was recorded as described above. For gene expression analysis, plants were harvested two hours after the fourth treatment, and shoot apical regions (containing
hypocotyl, SAM and leaf primordia smaller than 0.5 cm) or rosettes were isolated for analysis. For gene expression analysis on SARs of Landsberg erecta we proceeded as mentioned above but plants were sprayed after 13 days after sowing, as this ecotype flowers earlier. For measuring the effect of GA on petiole elongation, long-day grown plants were GA-treated as above on days 11, 13, 15 and 19 after sowing. Plants were harvested four days later, photographed and petioles were measured with ImageJ (National Institutes of Health). All experiments were repeated twice. Student T-tests were used to compare mean values.

Microscopy

Cryo-scanning electron microscopy (cryo-SEM) was performed by a modification of the method of Huang et al. (1994). Leaves were inserted into blocks, immediately frozen in liquid nitrogen and then loaded into a cryo-transfer unit. The leaves were either fracture for examination of internal tissues or left intact for analysis of epidermal cells and then gold-coated prior to cryo-SEM imaging. All samples were examined with a Cambridge S360 SEM (Cambridge, UK).

For analysis of meristem and leaf structure, samples were fixed, dehydrated in a graded ethanol series and infiltrated and embedded with LR White resin (London Resin Company). Semi-thin (2 μm) sections were stained with toluidine blue. Vein patterns were visualized by clearing cotyledons and third leaves with 70% ethanol. For histochemical localization of GUS activity we proceeded as described in Millar and Gubler (2005) and then cleared the tissue with a saturated chloral hydrate solution. Trypan blue staining was performed as described in Van Wees (2002).

For the visualisation of the aleurone layers, seeds were sowed on 0.6% agarose plates, stratified overnight and then incubated in the growth chamber for 6, 24, 30 and 48h. Then the aleurone layers were isolated and visualised as described in Bethke et al. (2007) with a Leica DMLB microscope. Each time point we scored 5 aleurone cells per seed in a total of 5 seeds per genotype (n=25) and we repeated the experiment twice. For determining the response of the aleurone to GA at 30°C, aleurone layers were isolated from seeds imbibed for 1-3h and then mounted on a slide with water or 30 μM GA4. Slides were placed in a humid chamber and
incubated at 30°C for five days prior to visualisation of the aleurones. We scored 20 seeds per genotype and treatment and we analysed three biological replicates.

**Morphometrical analysis of mir159ab leaves**

To estimate the size of the epidermal cells, we collected four to seven cryo-SEM images throughout the adaxial and abaxial surfaces of four leaves per genotype. We then counted the number of cells per SEM image and then divided the area of the picture by the number of cells to obtain average cell sizes.

We analysed a single cross-section of two Col-0 and two mir159ab fifth leaves with the program ImageJ. These cross-sections were perpendicular to the mid-vein and taken from the middle of the leaf. We measured the lengths of the adaxial and abaxial surfaces on the whole leaf-section, determined the adaxial/abaxial ratio of each leaf and then calculated the mean. To determine the number of epidermal cells we counted the number of adaxial and abaxial epidermal cells in the sections. To calculate the density of cells in the mesophyll we counted the number of mesophyll cells in the sections and divided it by the area of the sectioned leaf. Finally, we measured the area of 20 palisade and 20 spongy mesophyll cells per leaf and then calculated the mean to obtain mesophyll cell size.

**Microarray analysis**

Transcriptomic analysis was performed using Affymetrix GeneChip Arabidopsis Genome ATH1 microarrays. Three biological replicates were analysed for each genotype (Col-0, myb33.myb65, mir159ab) with each array representing a single biological replicate. We isolated total RNA as described (Chang et al., 1993) from the shoot apical region (shoot apices that included hypocotyl, meristem and leaf primordia shorter than 0.5 cm) of 15-day-old plants grown under long-day conditions. The quality of each total RNA sample was verified with an Agilent Bioanalyzer 2100 and the Agilent Eukaryotic total RNA Nano assay kit (Agilent Technologies, Inc., Santa Clara, CA). For each sample, biotinylated cRNA was prepared according to the standard Affymetrix single-amplification protocol from 5 µg total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). Following hybridizations, array quality was assessed using quality control metrics implemented in GCOS 1.4 (Affymetrix) and software procedures available in R/Bioconductor (Bioconductor version 2.4.0).
[Gentleman et al., 2004]) and at the website of The Centre for Bioinformation Science (http://cbis.anu.edu.au/software.html). Based on these metrics, the quality of all array hybridizations was assessed as satisfactory. CEL files were next imported into Partek Genomics Suite Version 6.3 (Partek Inc, St Louis, Mo, USA) and normalized by quantile normalization following RMA background correction with adjustment of probe cell intensities to correct for probe sequence effects. Probeset summarization was done with the median polish option. One-factor ANOVA analyses were carried out on the log2-transformed expression values of each of the 22810 probesets in Partek. Probesets with an uncorrected ANOVA p-value < 0.005 and with a fold change of 2 or greater between any two experimental groups were selected for further investigation.

All microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) at the following website [http://www.ncbi.nlm.nih.gov/geo/] and are accessible through GEO Series accession number (XXX).

**Gene expression analysis**

For determination of mRNA levels, RNA isolation and qRT-PCRs were performed as described in Allen et al. (2007) with primers listed in Table S3. Mature miR159 levels were quantified with the TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA) following manufacturer instructions. For these assays, RNA from shoot apices of short-day grown plants was extracted using TRIZOL (Invitrogen, Carlsbad, CA) and a 10 ng sample was retrotranscribed with TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) following the kit protocol. In each reaction, we included the stem-loop RT primers for either miR159a or miR159b and also the normalization gene sno101. 1.33 µl of RT-PCR product was used in 20 µl qRT-PCRs. Three technical replicates were done per sample and we analysed two different biological replicates.

GUS activity in transgenic plants was determined using the fluorogenic substrate 4-MUG as described in Jefferson et al. (1987) with 100 mg leaf discs. The fluorescence was measured using FLUOstar OPTIMA multidetection plate reader.
ACKNOWLEDGMENTS

We thank the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutant SALK_061355. Funding for the SIGnAL indexed insertion mutant collection was provided by the "National Science Foundation". Thanks are due to Cheng Huang for his assistance with SEM. Rod King and Jayne Griffiths kindly provided GA$_4$ and $ga1-3$ seeds respectively and much advice. We also thank Jose M. Barrero for his comments on the manuscript.

LITERATURE CITED


Lee EJ, Matsumura Y, Soga K, Hoson T, Koizumi N (2007) Glycosyl hydrolases of
cell wall are induced by sugar starvation in Arabidops. Plant and Cell Physiology 48: 405-413


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<th>Rice homologue up-regulated by GAMYB</th>
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### Up-regulated genes in *mir159ab*

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### Down-regulated genes in *mir159ab*

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<th>Rice homologue up-regulated by GAMYB</th>
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Table 1. Transcript profiling in *mir159ab* plants. Fold-change expression levels of select genes found to be mis-expressed in *mir159ab* according to micro-array analysis were measured by qRT-PCR in shoot apical regions (SAR), 28-day-old whole rosettes (WR), 3-day-old imbibed seeds (Seed), and...
flowers of mir159ab and in 28-day-old rosettes of transgenic lines carrying a mMYB33 construct (mMYB; Allen et al., 2007). The rice homologs of these genes that are regulated by GAMYB (Tsuji et al., 2006) in seeds (-S) or anthers (-A) are presented in the table with the percentage of similarity to the Arabidopsis counterparts and the alignment score calculated by the BLAST program in brackets (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).
Figure 1. Flowering-time of *myb33.myb65* and *mir159ab*. Flowering time under (A) long-day and (B) short-day conditions of Col-0 (wild-type), *myb33.myb65* and *mir159ab*. Error bars represent standard deviations (s.d.) and asterisks mark statistically significant changes.
Figure 2

(A) Short day conditions

Days to flower

(B) Relative mRNA levels

(C) Relative mRNA levels

(D) Petiole length of 23-day-old third leaves of plants grown under long-day conditions.

(E) mRNA levels of MYB33 and MYB65 in 32-day-old wild-type rosettes grown under short-day conditions. Black represents ethanol-treated and grey GA-treated plants. Error bars represent s.d. and asterisks mark statistically significant changes.

Figure 2. GA response of the miR159-GAMYB pathway in vegetative tissues. Effect of GA and ethanol treatments on (A) the flowering-time of wild-type and mutants under short-day conditions. (B) mRNA levels of MYB33 and MYB65 and the levels of mature miR159a and miR159b in wild-type SARs. (C) LEAFY, GIBBERELLIN 3 BETA-HYDROXYLASE 1 (GA3OX1) and SCARECROW-LIKE 3 (SCL3) mRNA levels in Col-0 and myb33.myb65 SARs grown under short-day conditions. (D) Petiole length of 23-day-old third leaves of plants grown under long-day conditions. (E) mRNA levels of MYB33 and MYB65 in 32-day-old wild-type rosettes grown under short-day conditions. Black represents ethanol-treated and grey GA-treated plants. Error bars represent s.d. and asterisks mark statistically significant changes.
Figure 3.

**MYB33** is strongly repressed by miR159 in vegetative tissues. Histochemical staining for GUS activity in 14-day-old seedlings of (A-B) **MYB33**:GUS after five-days of staining, (C) **mMYB33**:GUS after 16 hours of staining and (D) wild-type after five-days of staining. (E) Levels of **MYB33**:GUS (**MYB**) and **mMYB33**:GUS (**mMYB**) mRNA in five independent lines detected by qRT-PCR. (F) GUS activity in five independent **MYB33**:GUS (**MYB**) and **mMYB33**:GUS (**mMYB**) averaged lines. (G) Cartoon depicting the position of the primers used to quantify the mRNA of the transgenes. The primers span the miRNA target site of the **MYB33**:GUS construct and therefore only detect uncleaved mRNA. Error bars represent standard deviations (s.d.). Scale bars represent 100 (A) and 200 (B-D) µm.
Figure 4. Many genes up-regulated in mir159ab are GA-regulated in seeds and preferentially expressed in the aleurone. (A) Early GA induction in ga1-3 seeds of sixteen genes up-regulated in mir159ab. (B) Late GA response in ga1-3 seeds of fourteen up-regulated genes in mir159ab as determined by qRT-PCR. (C) Normalized expression levels for MYB33, MYB65 and the GA-induced genes in the aleurone and embryo. Numbers on top of the bars are absolute expression values in the aleurone. Data for A was obtained from AtGenExpress (Schmid et al., 2005) and data for C was obtained from Arabidopsis eFP browser (Winter et al., 2007).
Figure 5. Identification of GAMYB-like regulated genes in the seed. Visualization of MYB33:GUS expression in the (A) seed and (B) aleurone with dark field optics. (C) Fold changes in mRNA levels of the GA-responsive genes CP1, GASA1, BXL1, BXL2 and CP in myb33.myb65, myb101 and myb33.myb65.myb101 mutants as determined by qRT-PCR. (D) Genomic structure of the myb101 allele (SALK_061355). The conserved R2R3 MYB domain (R2R3) is represented in the gene. (E) MYB101 mRNA levels in myb101 mutant seeds. (F-H) mRNA levels of MYB33, MYB65 and MYB101 in Col-0 and ga1-3 seeds treated with GA. Error bars represent s.d.
Figure 6. The Arabidopsis *GAMYB-like* genes promote aleurone PCD. (A) Number of Protein Storage Vacuoles (PSV) per aleurone cell in wild-type and *myb33.myb65.myb101* (triple mutant) seeds at different time points during germination. N= 50 cells and s.d. range from 0 to 7.71. Error bars have been omitted for clarity. (B-C) Typical images of aleurone cells incubated at 30°C for five days without (B) and with GA (C). (D) Percentage of vacuolated aleurone layers after five days of incubation at 30°C with or without GA (n=20).
Figure 7. Trypan blue staining of third leaves of 13 day-old Col-0 and mir159ab plants. Necrotic rosette leaves from 6 week-old Col-0 plants were used as a positive control. Scale bar represent 2 mm.
Figure 8. *mir159ab* displays a hypertrophic Shoot Apical Meristem (SAM). Differential interference contrast (DIC) microscopy of cleared tissue from 4-day-old (A) wild-type and (B) *mir159ab* seedlings. SAM regions have been shaded. Median longitudinal cross-sections of 14-day-old (C) wild-type and (D) *mir159ab* SAMs. The red line is delimiting the L3 and subtending meristematic region. Scale bars represent 50 µm.
Figure 9

Figure 9. Alterations to leaf development in *mir159ab* plants. Transverse-sections of 24-day-old (A) wild-type and (B) *mir159ab* fifth leaves. Cryo-fracture of 24-day-old (C) wild-type and (D) *mir159ab* fifth leaves. Differences between wild-type and *mir159ab* concerning mesophyll (E) cell size and (F) cell density. SEM analysis of wild-type (G) adaxial and (H) abaxial, and *mir159ab* (I) adaxial and (J) abaxial surfaces. Epidermal (K) cell number and (L) cell size. Mid-vein cross-sections in (M) wild-type and (N) *mir159ab*. Venation pattern of 14-day-old (O) wild-type and (Q) *mir159ab* cotyledons and (P) wild-type and (R) *mir159ab* first leaves. P= phloem, X= xylem. Error bars represent s.d. and scale bars represent 50 (A-D, G-J, M-N) and 200 (O-R) µm.
Figure 10. Proposed model of the miR159-GAMYB regulatory pathway in *Arabidopsis thaliana*. GAMYB-like proteins are present in the aleurone, indicating low activity of miR159. In this tissue we hypothesize they transduce the GA-signal for the activation of GA-induced genes, which leads to nutrient secretion and the progression of PCD. Conversely, strong miR159 activity fully represses *MYB33* and *MYB65* in vegetative tissues, ensuring that aleurone-related genes remain inactive to allow the progression of growth. GA appears not associated with the miR159-GAMYB regulatory module in these tissues.