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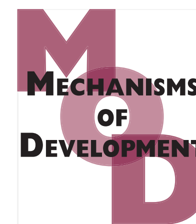

MicroRNA miR396 and *RDR6* synergistically regulate leaf development

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ABSTRACT

The microRNA (miRNA) miR396 regulates *GROWTH-REGULATING FACTORs* (*GRFs*), a plant specific family of transcription factors. Overexpression of miR396 causes a decrease in the *GRFs* that has been shown to affect cell proliferation in the meristem and developing leaves. To bring further insights into the function of the miR396 regulatory network we performed a mutant enhancer screen of a stable *Arabidopsis* transgenic line expressing 35S:*miR396b*, which has a reduction in leaf size. From this screen we recovered several mutants enhancing this phenotype and displaying organs with lotus- or needle-like shape. Analysis of these plants revealed mutations in *as2* and *rdr6*. While 35S:*miR396b* in an *as2* context generated organs with lotus-like shape, the overexpression of the miRNA in an *rdr6* mutant background caused more important developmental defects, including pin-like organs and lobed leaves. Combination of miR396 overexpressors, and *rdr6* and *as2* mutants show additional organ defects, suggesting that the three pathways act in concert. Genetic interactions during leaf development were observed in a similar way between miR396 overexpression and mutants in *RDR6*, *SGS3* or *AGO7*, which are known to participate in trans-acting siRNA (ta-siRNA) biogenesis. Furthermore, we found that miR396 can cause lotus- and pin-like organs *per se*, once a certain expression threshold is overcome. In good agreement, mutants accumulating high levels of *TCP4*, which induces miR396, interacted with the *AS1*/*AS2* pathway to generate lotus-like organs. The results indicate that the miR396 regulatory network and the ta-siRNA biogenesis pathway synergistically interact during leaf development and morphogenesis.

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1. Introduction

Plants, unlike animals, continue to produce new organs throughout their life cycle. The shoot apical meristem (SAM), located at the tip of the plant apex, contains a collection of stem cells that are ultimately responsible for all the above ground organs of the plants, such as leaves and stem. An excess of cells at the flanks of the meristem forms first a leaf primordia [reviewed in (Tsukaya, 2006)]. Originally a rod-like structure, the leaf primordia expands and flattens to form a lamina. During this process, a dorsoventral axis is generated and the two sides of the organ differentiate. The adaxial side (top) is anatomically prepared to capture light, while the abaxial side (bottom) is specialized in gas

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exchange [review in (Chitwood et al., 2007; Husbands et al., 2009)].

The dorsoventral axis of the leaf is established through a network of transcription factors and small RNAs. Members of the class III HD-ZIP family specify the adaxial cell fate, and loss of their functions causes abaxialized organs (Emery et al., 2003; McConnell and Barton, 1998; McConnell et al., 2001). A second pathway that contributes to adaxial differentiation includes the MYB-domain transcription factor *ASYMMETRIC LEAVES* 1 (AS1) (Byrne et al., 2000) in *Arabidopsis thaliana*, which is known as *PHANTASTICA* in other species (Tattersall et al., 2005; Waites et al., 1998). AS2, a member of the LOB/AS2 family interacts with AS1 to regulate cell differentiation (Lin et al., 2003; Xu et al., 2003). In turn, the abaxial side is specified by transcription factors of the *KANADI* (Eshed et al., 2001; Kerstetter et al., 2001), *YABBY* (Sawa et al., 1999; Siegfried et al., 1999) and *ARF* (Pekker et al., 2005) class. Together, these genes establish a complex network where synergistic interactions and mutual exclusions contribute to their final pattern of expression [reviewed in (Chitwood et al., 2007; Husbands et al., 2009)].

Two different small RNA pathways have been implicated in the precise pattern of expression of polarity genes. The first one involves the microRNA (miRNA) family miR165/166, which regulates the class III HD-ZIP genes and restricts their expression to the adaxial side of the leaf (Emery et al., 2003; Juarez et al., 2004; Kidner and Martienssen, 2004; Mallory et al., 2004b). A second pathway involves trans-acting short interfering RNAs (ta-siRNAs), which requires the additional activity of RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and DICER-LIKE 4 (DCL4) (Allen et al., 2005; Garcia et al., 2006; Peragine et al., 2004; Vazquez et al., 2004). Ta-siRNA formation triggered by the activity of miR390 in the context of ARGONAUTE 7 (AGO7) on the non-coding RNA *TAS3* in the adaxial side of the leaf generates a gradient of ta-siRNAs that in turn inhibit the expression of *ARF3/4* (Montgomery et al., 2008; Nogueira et al., 2007).

GROWTH-REGULATING FACTORs (*GRFs*) are a plant specific family of transcription factors, known to regulate leaf growth (Horiguchi et al., 2006; Horiguchi et al., 2005; Kim et al., 2003; Liu et al., 2009; Rodriguez et al., 2010). They act together with *GRF INTERACTING FACTORs* (*GIFs*) to control the organ development (Horiguchi et al., 2005; Kim and Kende, 2004). Mutations in *GRF1–3*, *GRF5* or *GIF1–3* reduce the size of the leaf (Horiguchi et al., 2005; Kim et al., 2003; Kim and Kende, 2004; Lee et al., 2009). In *Arabidopsis*, seven out of the nine *GRFs* are regulated by miR396 (Jones-Rhoades and Bartel, 2004). This miRNA is expressed at low levels in the meristem and young leaves, while it steadily accumulates during leaf development (Rodriguez et al., 2010).

Overexpression of miR396 causes a significant reduction of *GRF* expression and the cell number in leaves (Liu et al., 2009; Rodriguez et al., 2010), while expression of a *GRF2* genomic version insensitive to the miRNA repression causes an increase of leaf size (Rodriguez et al., 2010). MiR396 overexpression in the context of *as1* or *as2* mutants can lead to leaves with polarity defects (Wang et al., 2011), suggesting that other roles of the miR396 regulatory network could be uncovered in sensitized genetic backgrounds.

Here, we report an ethyl methanesulphonate (EMS) screen for enhancers of the 35S:*miR396b* leaf phenotype. We identified several mutants having leaves with lotus- or needle-like shape. Molecular characterization of these mutants revealed an interaction with pathways known to participate in leaf polarity. A strong synergistic effect was observed between the miR396 network and mutants involved in the biogenesis of ta-siRNAs during leaf development. Detailed analysis of miR396 overexpressors revealed that high levels of this miRNA can *per se* compromise leaf development to generate needle-like organs.

2. Results

2.1. A mutagenesis screen for enhancers of 35S:miR396b

High miR396 levels cause an evident reduction of the leaf lamina (Liu et al., 2009; Rodriguez et al., 2010), and also affect the size of the meristem (Rodriguez et al., 2010). Additionally, overexpression of miR396 in the context of a *gif1* mutant severely impairs the meristem integrity indicating that the miR396/*GRF* network has several roles during plant development (Rodriguez et al., 2010). To dissect the functions of the miR396/*GRF* network in leaf and meristem development, we mutagenized 35S:*miR396b* plants to search for enhancers of the leaf phenotype that do not compromise the function of the meristem.

We selected a homozygous and stable line overexpressing miR396b (35S:*miR396b* line #5) harboring smaller leaves than wild-type plants (approximately a 60% reduction) (Fig. 1B). Approximately 50.000 seeds were treated with EMS, allowed to self-pollinize, and then screened in the M2 population. Two week-old seedlings grown in plates were analyzed mainly focusing on the phenotype of the first pair of true leaves, which grow reproducibly under the assay conditions.

Interestingly, during the screening we found several plants harboring lotus- or needle-like organs (Fig. 1A–F). These leaf phenotypes are reminiscent of those caused by mutations in polarity genes [reviewed in (Chitwood et al., 2007)]. In many cases, mutations in genes that do not have an obvious effect on the dorsoventral axis *per se,* can have synergistic effects on leaf polarity once they are combined (Garcia et al., 2006; Li et al., 2005; Pekker et al., 2005; Xu et al., 2006). Two enhancers with lotus-like leaves and ten having stronger needle-like organs were chosen for further studies (Fig. 1A–F).

Mutations in *AS2* are known to enhance polarity defects in sensitized backgrounds (Fu et al., 2007; Kojima et al., 2011; Li et al., 2005; Xu et al., 2006), so we sequenced this gene in the 35S:*miR396b* mutant enhancers. We found that one of these mutant plants harboring lotus-like leaves had a mutation in *AS2* (Fig. 1C, D, G), which was validated by a cross to the characterized *as2–1* mutant (not shown). These results confirm that a mutation in *AS2* in the context of miR396 overexpression affects leaf patterning, in agreement with a recent report showing that 35S:*miR396* plants crossed to *as2* mutants have lotus-like leaves (Wang et al., 2011).

We found that the new mutation affecting *AS2* function changed a glycine at position 83 to a serine (Fig. 1C, D and G). This glycine is located in the leucine-zipper domain of AS2 and is largely conserved across the AS2/LOB family and also in different species (Fig. 1G) (Garcia-Ruiz et al., 2010; Iwakawa et al., 2002; Shuai et al., 2002). Our results highlight the importance of this region in AS2 function.

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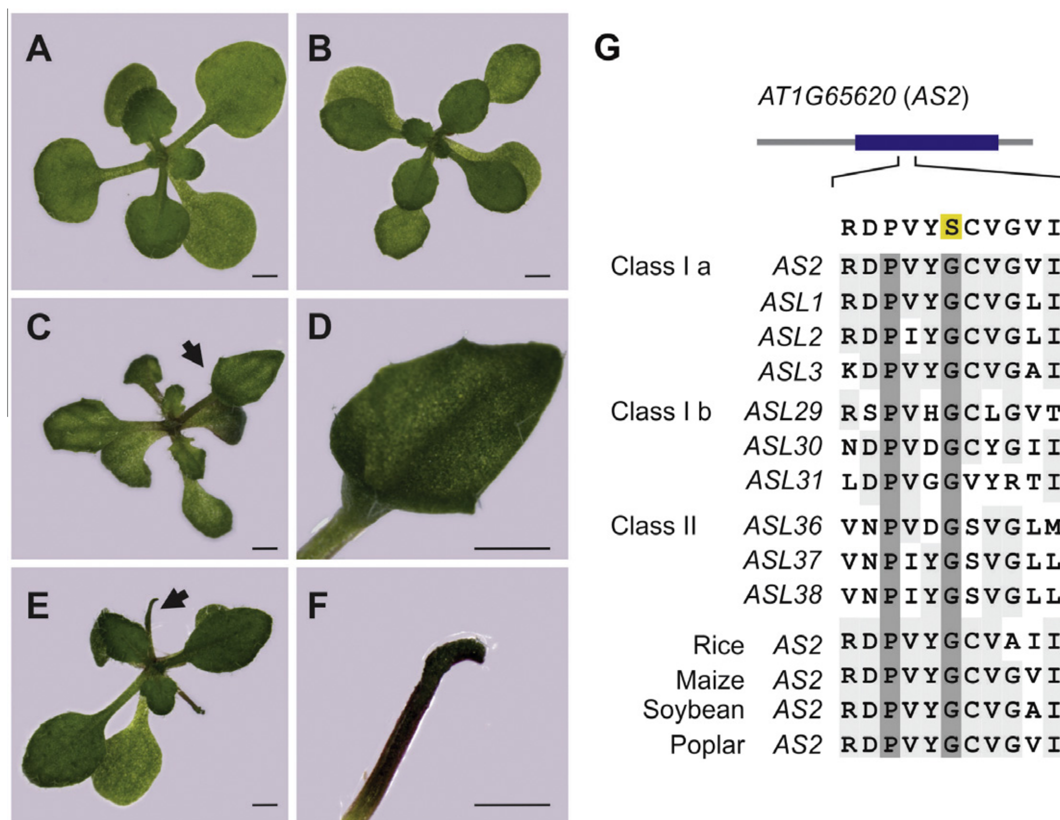

Fig. 1 – An enhancer screen of 35S:*miR396b* leaf phenotype. (A–B) 15-day-old wild-type (A) and 35S:*miR396b* (B) plants. (C–F) Mutations that enhance miR396 overexpression and cause lotus – (C–D) or needle – (E--F) like leaves. Arrows indicate lotus – (C) or needle – (E) like leaves, which are shown individually in panels (D) and (F), respectively. (G) Identification of a mutation in *AS2* as the gene responsible for the enhanced leaf phenotypes of 35S:*miR396b* shown in C–D. Aligment of residues 78 to 88 of members of AS2 family. Grey background indicates conserved amino acids. The mutation found in this screen is highlighted in yellow. Scale Bar: 2 mm.

2.2. *Overexpression of miR396 in rdr6 mutants generates needle-like leaves*

We were interested in identifying the mutations causing the stronger needle-like organs in the 35S:*miR396b* plants. Other small RNAs known to regulate leaf polarity belong to the ta-siRNA pathway, so we decided to test a possible interaction with miR396. The biogenesis of ta-siRNAs requires the seminal activity of the AGO7-miR390 complex on *TAS3* non-coding RNA, and the additional participation of RDR6, SGS3 and DCL4 (Allen et al., 2005; Vazquez et al., 2004). So, we decided to sequence *RDR6* from the 35S:*miR396b* enhancers.

Interestingly, we found that one of the enhancers with needle-like leaves harbored a mutation causing a premature stop codon in *RDR6* (Fig. 2A–D). As a consequence, the truncated protein contained 392 aminoacids instead of the usual 1196. Crosses between the 35S:*miR396b* enhancer and the known *rdr6–11* mutant confirmed the nature of this mutation (not shown). Furthermore, transformation of *rdr6–11* with a 35S:*miR396b* vector caused a reduction of the leaf lamina and in strong cases pin-like organs (Fig. 2E). Still, the reduced lamina showed its characteristic features on the adaxial side, such as the presence of trichomes (Fig. 2E, inset).

Mutations in *as2 per se* have stronger leaf developmental defects than those observed in *rdr6* mutants; however we observed that the overexpression of miR396 in the context of *rdr6* caused much stronger defects than in *as2* (Fig. 1 and Fig. 2), suggesting synergistic interactions between the miR396 network and *RDR6*.

2.3. *miR396 acts synergistically with the ta-siRNA biogenesis pathway to regulate leaf development*

To test whether the defects caused by the *rdr6* mutation in miR396 overexpressors are consequence of a deficiency in the ta-siRNA pathway, we overexpressed miR396 in the context of *rdr6*, *sgs3* and *ago7* (*zip-2*) mutants. Overexpression of miR396b in *rdr6* mutant background caused a high percentage (~70%) of independent transgenic lines with needle-like leaves (Fig. 3A and B).

Transformation of *sgs3* and *zip-2* mutants with 35S:*miR396b* generated plants displaying needle-like leaves in ~70% and ~55% of the transgenic lines, respectively (at least 50 primary transgenic plants were analyzed in each case) (Fig. 3A and B). Needle-like organs were usually observed in the first pair of leaves, while other leaves generated later in development displayed asymmetric lobes (Fig. 3C and

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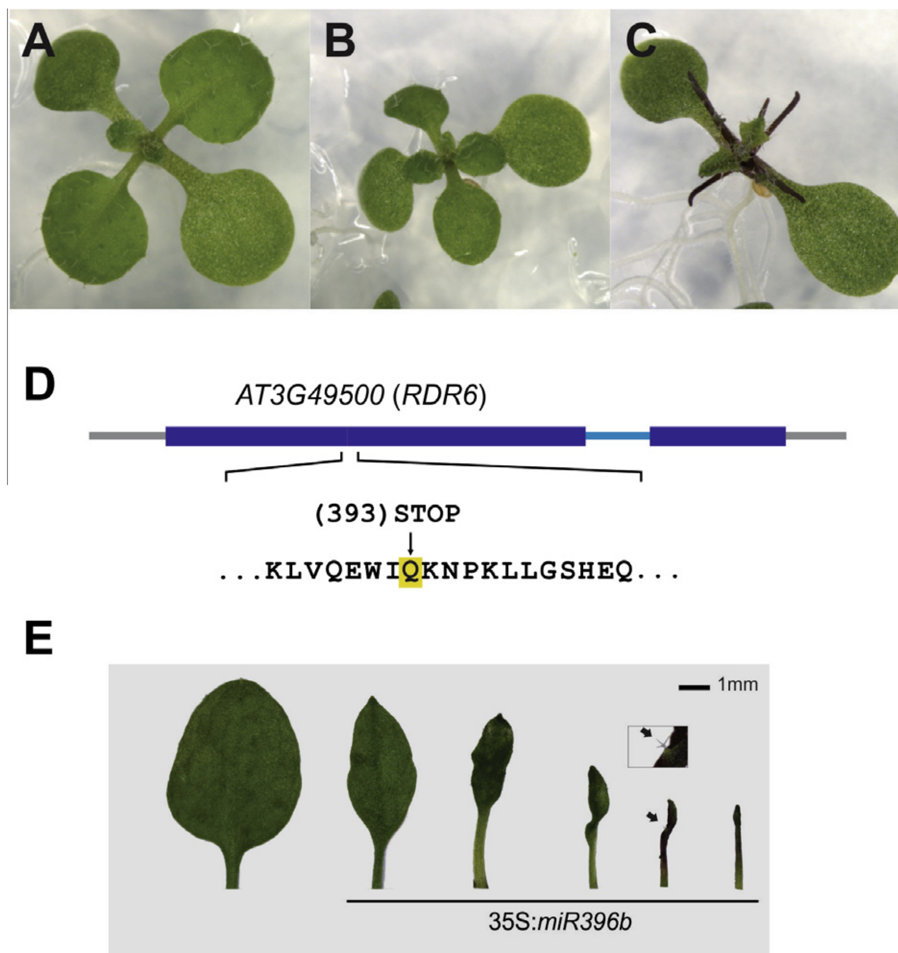

Fig. 2 – Identification of a mutation in *rdr6* causing needle-like leaves in 35S:*miR396b* plants. (A–C) 10-day-old rosettes of wild-type (A), 35S:*miR396b* (B) and a 35S:*miR396b* enhancer mutant displaying needle like leaves (C). (D) Diagram showing *RDR6* gene structure. The position of the nonsense mutation identified in the lane 35S:*miR396b* enhancer mutant shown in (C) is highlighted in yellow. (E) Overexpression of miR396b in *rdr6–11*. Effect of 35S:*miR396b* on leaf 1 of different primary transformants. The arrow and inset show a trichome in the reduced adaxial side of the lamina.

D). These results show a synergistic interaction between the miR396 network and the ta-siRNA pathway during leaf development.

2.4. *Interactions between AS2, miR396 and the ta-siRNA pathway*

It is known that simultaneous mutations in *as2* and *rdr6* cause defects in the polarity of developing leaves (Xu et al., 2006). Therefore, we analyzed the interaction between *as2* and *rdr6* mutants, and 35S:*miR396b* plants. The combination of these mutant and transgenic plants severely affected leaf development (Fig. 4A–F).

We found that *rdr6* mutants crossed to *as2* (Fig. 4E) have stronger developmental defects than *as2* plants crossed to 35S:*miR396b* (Fig. 4D). Our previous results also showed that 35S:*miR396b* had stronger developmental defects when combined to *rdr6* mutants (Fig. 2) than in the context of *as2* (Fig. 1). In *as2* × *rdr6* double knock-outs, leaf blade was severely affected, mainly in the first leaves where lamina is mostly absent. In *as2* × *rdr6* × 36S:*miR396b*, lamina absence occurred in almost all rosette leaves, and the organs mainly consisted of midvein and secondary veins (Fig. 4D, F). These results highlight a main effect of *rdr6* likely through the generation of ta-siRNAs in leaf development that is revealed when the mutant is combined with other regulators of organ growth.

2.5. *Quantitative effects caused by miR396 levels on leaf development*

Previous results have shown that 35S:*miR396b* plants cause smaller leaves (Liu et al., 2009; Rodriguez et al., 2010). We were, however, interested to test whether high levels of miR396 *per se* could generate lotus- or needle-like organs. To do this, we analyzed primary transformants harboring a 35S:*miR396a* or 35S:*miR396b* transgene. In general, we observed that developmental defects of plants harboring 35S:*miR396a* were stronger than those of 35S:*miR396b* (Fig. 5A). At least 90% of the transgenic plants ($n = 50$) overexpressing miR396a had obvious leaf developmental defects, while 70% was observed in the case of miR396b (Fig. 5A).

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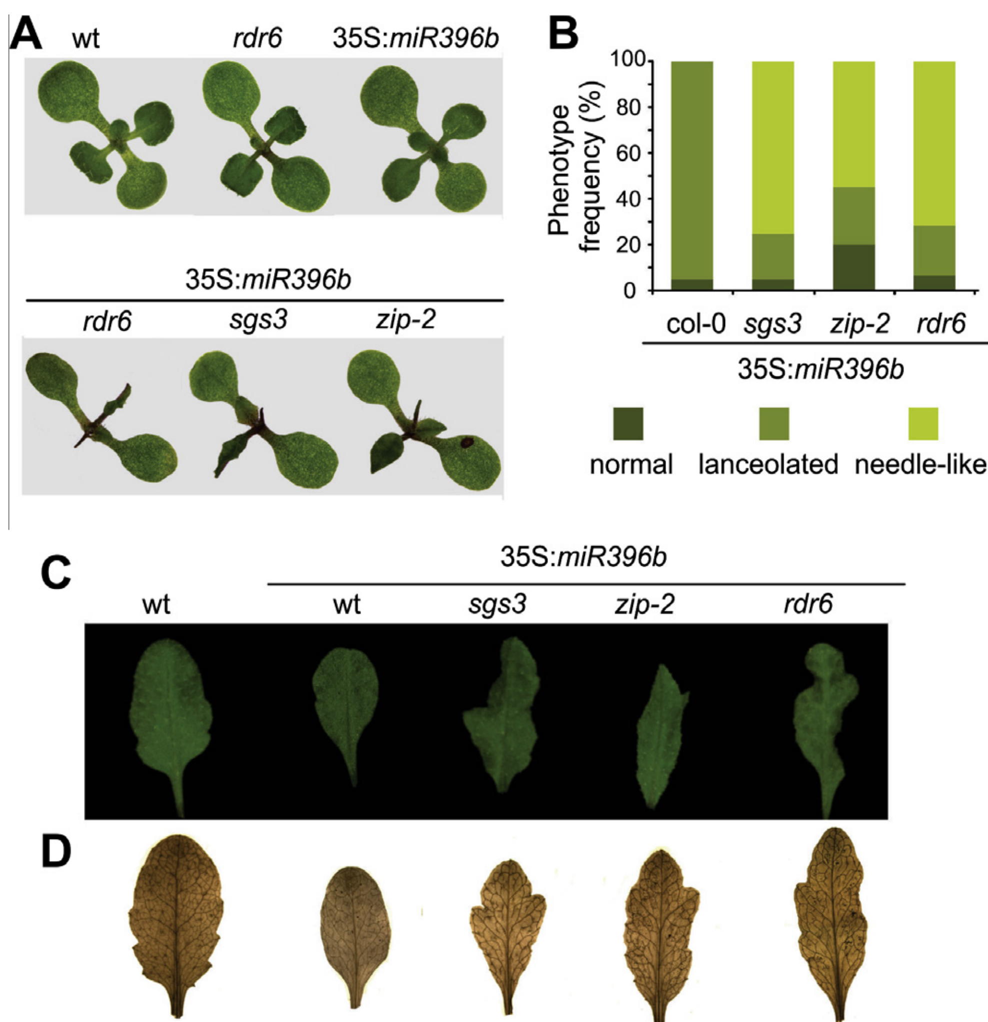

Fig. 3 – Leaf developmental defects caused by high levels of miR396 in ta-siRNA biogenesis mutants. (A) Representative phenotypes of ta-siRNAs biogenesis mutants harboring a 35S:*miR396b* transgene. *zip-2* is an *ago7* mutant. (B) Frequency of the different leaf developmental defects in the transgenic lines depicted in (A). At least fifty plants per construct were analyzed in each case. (C-D) Developmental defects in leaf #7 in ta-siRNAs biogenesis mutants overexpressing miR396b. Leaves were cleared in chloral hydrate to show the vasculature in (D). Note the lobes caused by miR396 overexpression in ta-siRNAs biogenesis mutants.

Most interesting, we found that transgenic plants harboring a 35S:*miR396a* vector had lotus- or needle-like organs in more than 30% of the cases (Fig. 5A and C). Differences in the strength of the phenotypes caused by expressing members of the same miRNA family have already been reported before (Mallory et al., 2004a; Palatnik et al., 2007; Schwab et al., 2005).

To test whether the enhanced phenotype of 35S:*miR396a* was due to a higher accumulation of the miRNA, we performed small RNA blots from pools of primary transgenics harboring 35S:*miR396a* or 35S:*miR396b* (Fig. 5B). We found that the vector expressing the *miR396a* accumulated more small RNA than 35S:*miR396b*, in good agreement with the stronger phenotypes observed for 35S:*miR396a* (Fig. 5A–C).

Then, we measured miR396 levels in 35S:*miR396a* plants with moderate and strong leaf defects (Fig. 5C and D). We observed that transgenics with lotus- or needle-like leaves accumulate two times more miR396 than those displaying only a reduction in leaf lamina (Fig. 5C and D). Interestingly, 35S:*miR396a* lines with moderate leaf phenotypes (Fig. 5C and D) had a similar increase in miR396 levels as the lines expressing 35S:*miR396b* (Fig. 5B), in good agreement with a quantitative effect of miR396 in leaf development.

As miR396 affects the SAM and leaf development (Rodriguez et al., 2010), we decided to express *miR396a* specifically in the leaf primordia using an *AS1* promoter (AS1:*miR396a*) (Byrne et al., 2000). We observed also in this case that many plants had lotus- and needle-like organs (Fig. 5A). Altogether, these results show that a moderate increase of miR396 levels in developing leaves compromises the lamina expansion, while high levels of the miRNA cause strong developmental defects and the generation of lotus- and needle-like organs.

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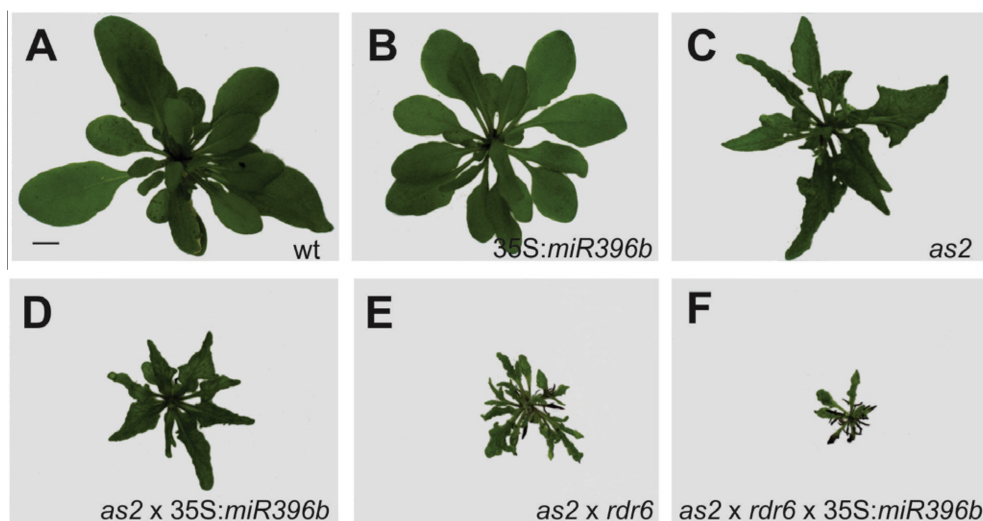

Fig. 4 – Interactions between 35S:*miR396b*, and *rdr6* and *as2*. (A-F) Rosettes from 4-week-old plants of wild-type (A), 35S:*miR396b* (B), *as2* (C), *as2* × 35S:*miR396b* (D), *as2* × *rdr6* (E), and 35S:*miR396b* × *as2* × *rdr6* (F). Scale Bar: 1 cm.

2.6. *Expression pattern of GRF genes*

Analysis of transgenic plants with high levels of miR396 did not reveal obvious changes in epidermal cell morphology attributable to an abaxialization of the leaf (data not shown). Then, we analyzed the expression of polarity genes at the molecular level by RT-qPCR in 35S:*miR396b* plants (Fig. 6B). The 35S:*miR396b* line used in our screening expressed approximately 7 times more miRNA than wild type (Fig. 6A). We found a small reduction in the transcript levels of several class III HD-ZIP transcription factors, such as *REVOLUTA* (*REV*), *PHAVOLUTA* (*PHV*) and *PHABULOSA* (*PHB*) (Fig. 6B). We also found changes in the transcript levels of other genes involved in the formation of the dorsoventral patterning of the leaf (Fig. 6B). These changes might be a consequence of the accelerated leaf differentiation of the *35S:miR396* leaves caused by a reduction in cell proliferation (Rodriguez et al., 2010; Wang et al., 2011), which in turn can affect genes expressed during early leaf development. In good agreement with this possibility, global analysis of *35S:miR396b* microarrays (Rodriguez et al., 2010) using a previously described polarity index (Sarojam et al., 2010) indicated that they are similar to wild-type plants (not shown).

Next, we analyzed published microarray data performed on plants with abaxialized or adaxialized leaves due to the ectopic expression of *KAN2* (*ANT:KAN2*) and *PHD* (*phd-1D* mutant), respectively (Malitsky et al., 2008). Interestingly, we observed that *GRFs* have higher expression levels in adaxialized leaves (*phd-1D*), while they are repressed in plants ectopically expressing *KAN2* (Fig. 6C). These results are in agreement with the pin-like structures observed in *35S:miR396b* plants, and might suggest that *GRFs* are downstream of genes establishing the adaxial side, such as *PHB*.

We analyzed the expression of a previously described *GRF2* reporter (*GRF2:wtGRF2-GUS*) (Rodriguez et al., 2010) in young developing leaves. In 4 day-old seedlings we observed that GUS activity preferentially accumulated in the adaxial side of the leaves (Fig. 6D). This pattern of expression was obtained when the seedlings were incubated with the staining solution for a short period of time (4 h), while longer incubations (16 h) lead to a complete staining of the primordia (not shown). Taken together, these results indicate a link between the networks establishing the dorsoventral axis of the leaf and the *GRFs*.

2.7. *Control of leaf development by TCP4, a positive regulator of miR396*

Previous work has shown that TCP4 controls miR396 levels (Rodriguez et al., 2010). TCPs are plant specific transcription factors comprising 24 different members in *Arabidopsis thaliana* (Martin-Trillo and Cubas, 2010). Five of them, including *TCP4*, are regulated by miR319 (Palatnik et al., 2003). They have been implicated in the control cell proliferation and differentiation in plants (Efroni et al., 2008; Nath et al., 2003; Ori et al., 2007; Palatnik et al., 2003).

The *soj8* mutant contains a point mutation in the miRNA target site of *TCP4* diminishing the interaction with miR319 and over-accumulating *TCP4* (Palatnik et al., 2007). This mutant has leaves with less cells, which correlates with high miR396 levels (Rodriguez et al., 2010), but does not have lotus- or needle-like organs. To test whether high levels of *TCP4* can interact with the *AS1/AS2* pathway, we crossed *soj8* to the *as1* mutant (Fig. 7A–E). The resulting plants of the cross *soj8* × *as1* had relatively small leaves (Fig. 7D). Most interesting, lotus-like organs can also be found among the first leaves (Fig. 7E). These results reinforce the notion that *TCP4* and miR396 act sequentially in the regulation of leaf development.

3. Discussion

Small RNAs play an essential role in the regulation of gene expression in plants. Both miRNAs and ta-siRNAs originate from endogenous loci and control the levels and activity of other longer RNAs of the cell. They can be distinguished by their biogenesis pathways and the genetic requirements for

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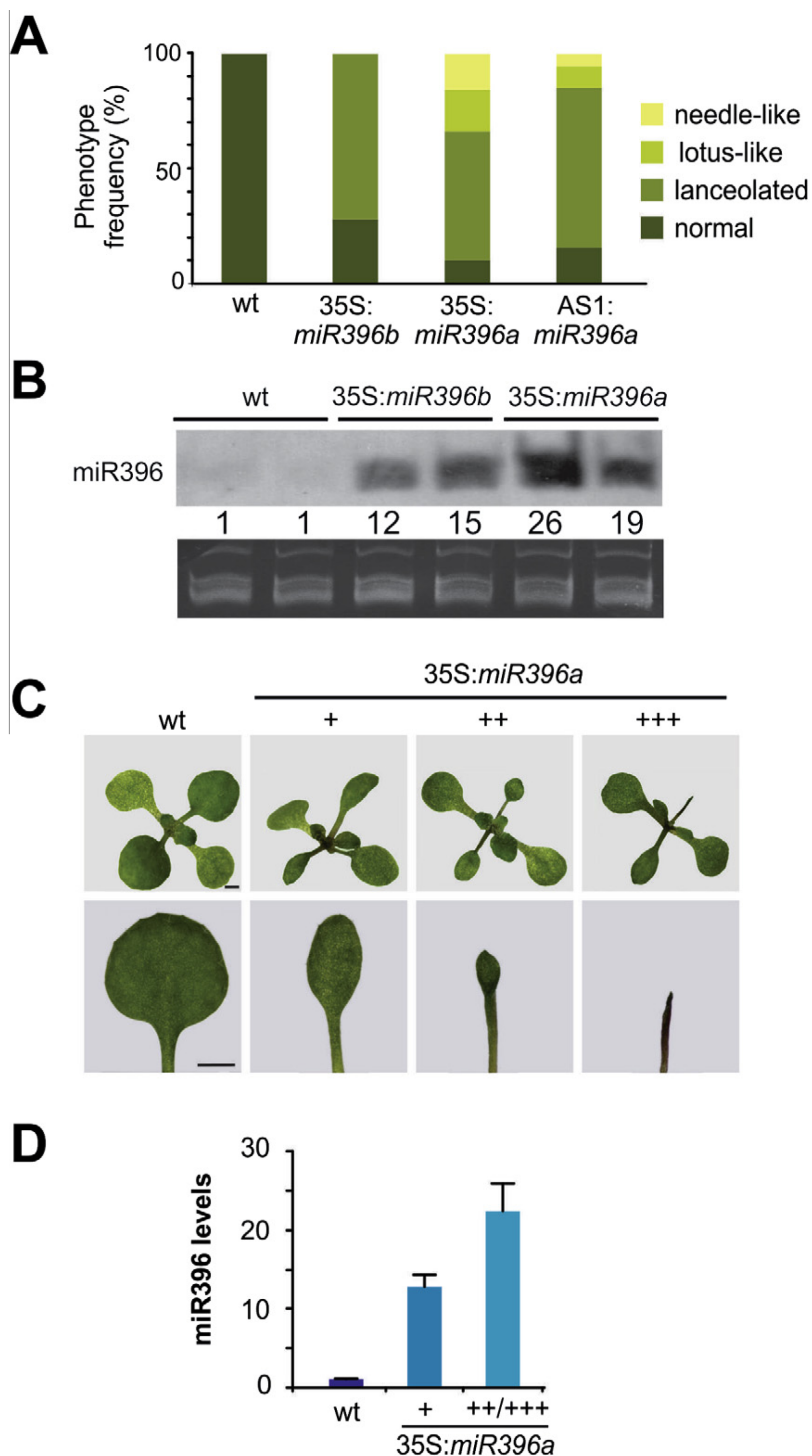

Fig. 5 – Leaf developmental defects caused by high miR396 levels. (A) Frequency of leaf developmental defects in independent transgenic lines expressing 35S:*miR396a*, 35S:*miR396b* and AS1:*miR396a*. At least fifty independent transgenic plants were analyzed in each case. (B) Expression of miR396 in wild-type and 35S:*miR396a* or 35S:*miR396b* transgenic plants of 15-day-old plants. RNA from pools of 20 independent plants chosen randomly was loaded in each lane. The numbers below the blot indicate the relative amount of miR396 in each case. (C) Rosette and first leaf phenotypes of 15-day-old plants of wild-type, and 35S:*miR396a* transgenic plants displaying lanceolate (+), lotus- (++) or needle- (+++) like leaves. Scale Bar: 2 mm. (D) Quantification of mature miR396 in 15-day-old plants of wild type and 35S:*miR396a* transgenic plants by RT-qPCR. The expression levels were normalized to the wild-type. The data shown are mean ± s.e.m. of three biological replicates.

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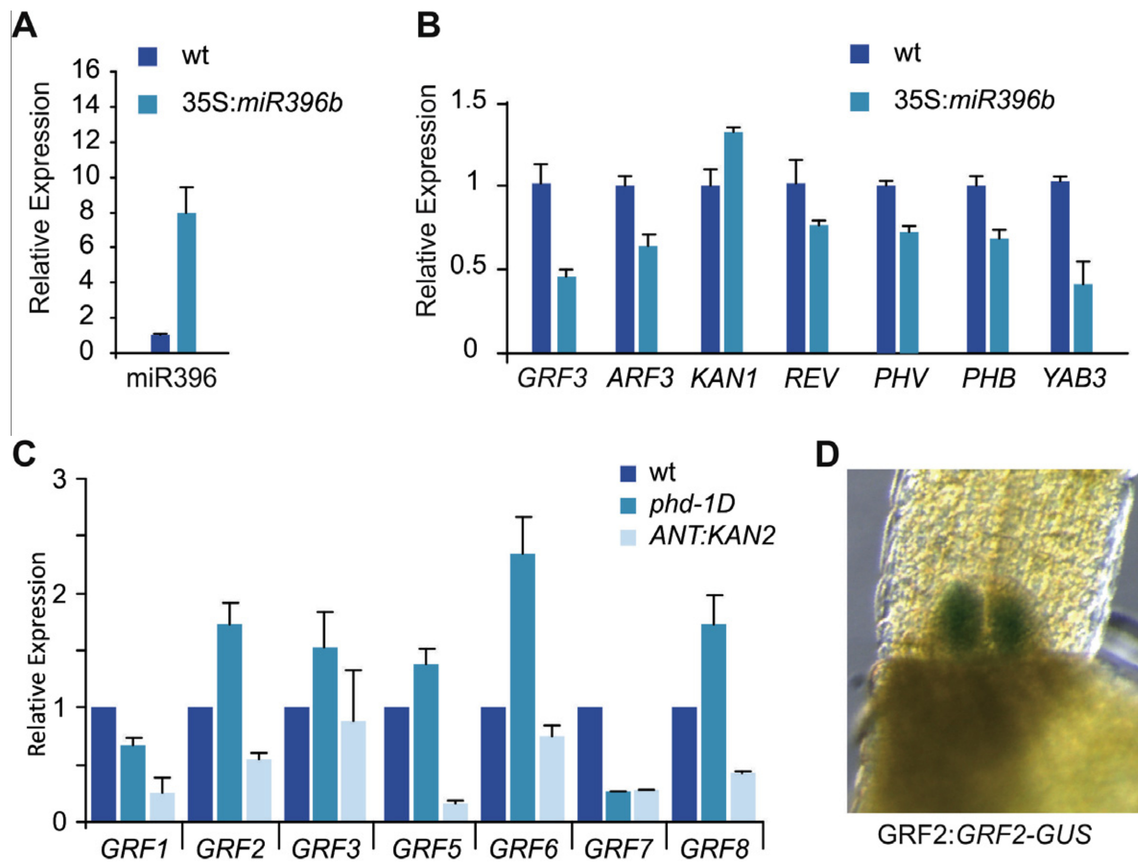

Fig. 6 – Expression of *GRFs* and polarity genes in different genetic backgrounds. (A-B) Quantification of miR396, *GRF3* and polarity genes in apices of 10 day-old seedlings by RT-qPCR. The expression levels were normalized to wild-type plants. The data shown are mean ± s.e.m. of three biological replicates. Differences between transgenic and wild-type plants are significant with the exception of *KAN1*, as determined by *Student's t-test* ($P < 0.05$). (C) Differential mRNA expression levels between the adaxial and abaxial leaf domains for seven *GRF* transcription factors. Data obtained from microarrays of ANT:*KAN* transgenic plants and *phb-1D* mutants (Malitsky et al., 2008). *GRF4* and *GRF9* are not present in the ATH1 array. (D) GUS staining of 4 day-olds GRF2:*wtGRF2-GUS* plants. The staining was performed during 4 h.

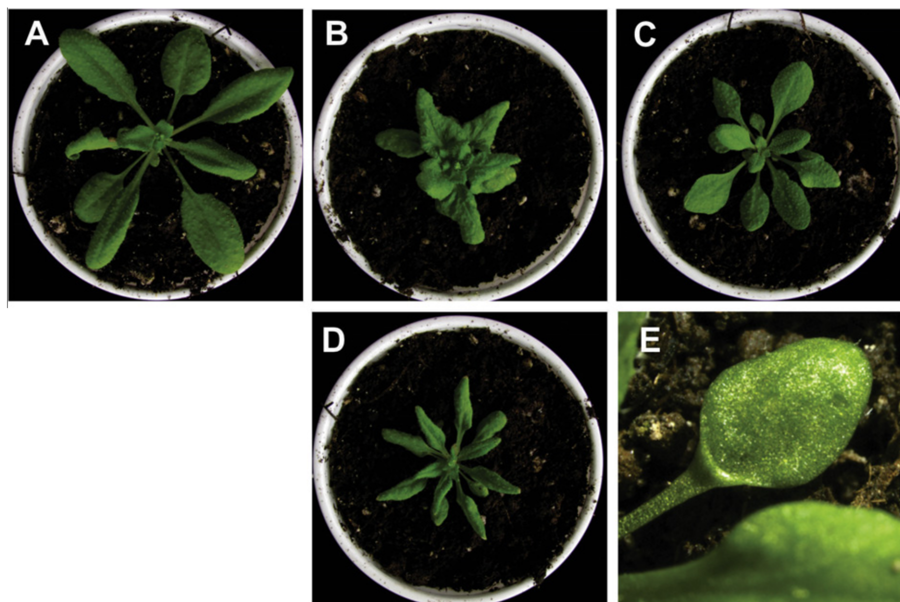

Fig. 7 – Interaction between *soj8* and *as1*. (A-D) 3-week-old plants of wild-type Col-0 (A), *as1* (B), *soj8* (C) and *soj8* × *as1* (D). (E) Lotus-like organs observed in the first leaves of *soj8* × *as1*.

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their production (Chapman and Carrington, 2007; Vaucheret, 2006). Many miRNAs, especially those that are evolutionary conserved, regulate plant developmental processes (Axtell and Bartel, 2005; Jones-Rhoades et al., 2006), and the pathways regulated by miRNAs tend to integrate the activity of several small RNAs (Rubio-Somoza and Weigel, 2011). Among these cases are the regulation of developmental timing in Arabidopsis by the sequential activity of the miR156 and miR172 networks (Wang et al., 2009; Wu et al., 2009), or the control of cell proliferation by miR319 (Nath et al., 2003; Palatnik et al., 2003) and miR396 (Rodriguez et al., 2010).

The establishment of the dorsoventral axis of the leaf integrates the activity miR165/miR166 family of miRNAs and the miR390-generated ta-siRNAs, which are expressed in opposing gradients along this axis [reviewed in (Chitwood et al., 2007; Husbands et al., 2009)]. The results presented here show that a genetic interaction exists also between the ta-siRNAs biogenesis machinery and the miR396 network during leaf development.

MiR396 can potentially regulate several target genes in *Arabidopsis thaliana*, however the only evolutionary conserved ones are the *GRFs* (Debernardi et al., 2012). The *GRFs*, as well as their co-activators have been implicated in the control of cell proliferation in developing leaves (Horiguchi et al., 2006; Horiguchi et al., 2005; Kim et al., 2003; Kim and Kende, 2004) and the meristem (Lee et al., 2009; Rodriguez et al., 2010). Recently, it has been shown that down-regulation of the *GRFs* by miR396 (Wang et al., 2011) or mutation in the *GRF* co-activator *GIF1* (also known as *AN3*) (Horiguchi et al., 2011) can generate lotus-like leaves in the context of *as2* or *as1* mutants.

The 35S:*miR396b* enhancer screen performed here identified new alleles of *as2* and *rdr6*. While mutations in *as2* could only cause lotus-like leaves in miR396b overexpressors, the loss-of-function of *rdr6* had a more pronounced impact on leaf development, generating pin-like or lobed organs. Interestingly, mutations in *rdr6* alone have minor defects in leaf development, in contrast to the more severe defects observed in *as2* mutants, indicating that there are synergistic interactions between the miR396 regulatory network and *rdr6*.

Genetic interactions between *RDR6-SGS3-AGO7* and the *AS1/AS2* pathway occur during leaf morphogenesis and both pathways cooperatively repress *KNAT1* and miR165/166 expression (Li et al., 2005; Xu et al., 2006). We observed that the combination of miR396 overexpressors, and *rdr6* and *as2* mutants showed additional leaf defects in each case, so that these three networks act at least partially in parallel during leaf development. *RDR6* is known to participate in several RNA-mediated silencing pathways (Mallory et al., 2008; Vaucheret, 2006). However, the similar results that we obtained after overexpressing miR396 in *sgs3* and *zip-2* mutants point towards the participation of the ta-siRNA pathway in the interaction with miR396.

Furthermore, we have found that very high levels of miR396 can also generate lotus- and pin-like structures *per se*, showing that miR396 has quantitative effects in leaf development and that lotus- or pin-like structures can be generated when the miRNA levels overcome a certain threshold. In good agreement, high *TCP4* levels which are known to induce miR396 and repress the *GRFs* can also cause lotus-like leaves in plants compromised in the *AS1/AS2* pathway.

It has been suggested that altered cell proliferation patterns could generate pin-like leaves (Horiguchi et al., 2011; Wang et al., 2011; Yuan et al., 2010). The effects of miR396 overexpression, a known regulator of cell proliferation (Rodriguez et al., 2010; Wang et al., 2011), are in good agreement with this possibility. The preferential expression of a *GRF2* reporter in the adaxial side of the organ and the changes in the expression of *GRFs* in leaves with polarity defects suggest that *GRFs* are downstream of genes involved in the formation of the dorsoventral axis. In this context, it might be plausible that class III HD-ZIP transcription factors control *GRF* expression.

In turn, the modification of the *GRF* levels might affect the expression of polarity genes due to an earlier differentiation of the leaf primordia; however it cannot be discarded that the *GRFs* participate in the complex regulatory networks that establish the polarity of the leaf. Genes directly regulated by the GRFs are largely unknown at the moment. It would be interesting to identify direct targets of these transcription factors by genome-wide approaches and analyze whether there are specific connections with polarity genes.

4. Experimental procedures

4.1. *Plant Material and leaf analysis*

Arabidopsis thaliana ecotype Col-0 was used for all experiments. Plants were grown either on soil or on MS medium in long photoperiods (16 h light/8 h dark) at 23 °C. For EMS mutagenesis, 35S:*miR396b* seeds were treated with 0.2% EMS over-night. Seeds were collected from 10,000 single fertile M1 plants and progeny were screened for leaf development defects. DNA was extracted using CTAB protocol (Weigel and Glazebrook, 2009). To analyze the vein pattern, leaves were fixed with FAA and cleared with chloral hydrate solution. Pictures were then taken under dark field illumination in a dissecting microscope. Seeds of *sgs3–11* and *zip-2* were kindly provided by S. Poethig (University of Pennsylvania, Philadelphia). *as1–1*, *as2–1* and *rdr6–11* alleles were obtained from the Arabidopsis stock center, and *soj8* has been described previously (Palatnik et al., 2007).

4.2. *Transgenes*

Previously described plants expressing 35S:*miR396b* (Rodriguez et al., 2010) were analyzed and a stable line expressing the transgene was selected for the EMS screen. 35S:*miR396a* construct was generated fusing 400 bp of *MIR396a* precursor to 35S promoter in the *pCHF3* binary plasmid, which contains a 3SS viral promoter (Jarvis et al., 1998). This viral promoter was replaced by the *AS1* promoter (4.7 kb), which has been used previously (Wang et al., 2008), to generate the vector AS1:*miR396a*.

4.3. *Expression Analysis*

RNA was extracted using TRIzol reagent (Invitrogen). Briefly, 1.0 μg of total RNA was treated with RQ1 RNase- free DNAse (Promega). Then, first-strand cDNA synthesis was car-

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ried out using SuperScriptTM III Reverse Transcriptase (Invitrogen) with the appropriate primer. PCR reactions were performed in a Mastercycler® ep realplex thermal cycler (Eppendorf) using SYBRGreen I (Roche) to monitor dsDNA synthesis. For each genotype, quantifications were made in triplicate, on two independently obtained cDNA samples. The relative transcript level was determined for each sample, normalized using *PROTEIN PHOSPHATASE 2A* cDNA level (Czechowski et al., 2005). Primers for real-time PCR were as follows: 5′-CCTGCGGTAATAACTGCATCT-3′ and 5′-CTTCACTTAGCTCCACCAAGCA-3′ for *PP2A*; 5′-GGGGTACCTAACCCAACCAACAAGAC-3′ and 5′-GGTCGACCTAAGCTTCATCGTAGATCC-3′ for *GRF3*; 5′-ACAACTGGTCCCAAGAGAAGCA-3′ and 5′-ACCTTGCAAGACCCTCTGGAAT-3′ for *ARF3*; 5′-CACGAGAGACATGGCCATTAAG-3′ and 5′-CCTCTTTGCTTGATCATTCGACC-3′ for *KAN1*; 5′-AAGTTACAGTCATCACTTAGGCTC-3′ and 5′-GGTTCGCAAACATGAACACTGG-3′ for *REV*; 5′-GGCGAGACAGTATGTGAGGAACG-3′ and 5′-AGCTCCAAACAGATCTGCACCT-3′ for *PHB*; 5′-CGTCAGTACGTGCGAAGCATAGTAG-3′ and 5′-CAGATCCAAAGAGATCTGCACCAG-3′ for *PHV*; and 5′-TCGCAACGGAAGATCAGTGGA-3′ and 5′-GGGTTGCCTGCCTTTATACGTT-3′ for *YAB3*. Mature miRNA miR396 levels were determined by stem-loop RT-qPCR as described previously (Chen et al., 2005). The sequences of the oligonucleotides used were: 5′-GTCTCCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGGAGACMAGTTC-3′ for retrotranscription and 5′-GGCGGTTCCACAGCTTTCTT-3′ and 5′-TGGTGCAGGGTCCGAGGTATT-3′ for RT-qPCR. To visualize the activity of GUS reporters, 4 days-old transgenic plants were subjected to 4 h GUS staining.

4.4. *Small RNA blots*

Total RNA was resolved on 17% polyacrylamide gels under denaturing conditions (7 M urea). Blots were hybridized using digoxigenin end-labelled locked nucleic acid (LNA) oligonucleotide probes designed against miR396 (Exiqon, Denmark).

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