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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.

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.