MicroRNA miR396 Regulates the Switch between Stem Cells and Transit-Amplifying Cells in Arabidopsis Roots

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To ensure an adequate organ mass, the daughters of stem cells progress through a transit-amplifying phase displaying rapid cell division cycles before differentiating. Here, we show that Arabidopsis thaliana microRNA miR396 regulates the transition of root stem cells into transit-amplifying cells by interacting with GROWTH-REGULATING FACTORS (GRFs). The GRFs are expressed in transit-amplifying cells but are excluded from the stem cells through inhibition by miR396. Inactivation of the GRFs increases the meristem size and induces periclinal formative divisions in transit-amplifying cells. The GRFs repress PLETHORA (PLT) genes, regulating their spatial expression gradient. Conversely, PLT activates MIR396 in the stem cells to repress the GRFs. We identified a pathway regulated by GRF transcription factors that represses stem cell-promoting genes in actively proliferating cells, which is essential for the progression of the cell cycle and the orientation of the cell division plane. If unchecked, the expression of the GRFs in the stem cell niche suppresses formative cell divisions and distorts the organization of the quiescent center. We propose that the interactions identified here between miR396 and GRF and PLT transcription factors are necessary to establish the boundary between the stem cell niche and the transit-amplifying region.

INTRODUCTION

Both plants and animals rely on stem cells for the generation of the different cell types that constitute their body parts. Stem cells are located within specific cellular contexts referred to as stem cell niches (SCNs). As stem cells divide slowly, their progeny generally undergo rapid, transient amplifying cell divisions to ensure that there are enough cells for proper organ growth before differentiation. Cells undergoing this process are called transit-amplifying cells (TACs) (Koster and Roop, 2007; Scheres, 2007; Lui et al., 2011; Heidstra and Sabatini, 2014).

In plants, the root SCN is formed by the quiescent center (QC) and the adjacent stem cell initials (Petricka et al., 2012), which are specified by two parallel pathways: the PLETHORA (PLT) and SHORTROOT (SHR)/SCARECROW (SCR) pathways (Petricka et al., 2012; Heyman et al., 2014).

SHR and SCR encode members of the GRAS family of transcription factors (named after the first three members, GIBBERELLIC-ACID INSENSITIVE, REPRESSOR of GAI, and SCR (Pysh et al., 1999)). SHR expressed in the stelar moves into the QC and cortex/endodermal initials (Nakajima et al., 2001) to activate SCR expression (Levesque et al., 2006). In turn, SCR maintains QC and stem cell identity (Sabatini et al., 2003), in part by inducing the expression of WUSCHEL-RELATED HOMEBOX5, a QC-specific gene (Sarkar et al., 2007).

PLT proteins are expressed in a gradient along the longitudinal axis of the root that is established by a combination of cell-to-cell movement and mitotic segregation of proteins from a narrow transcriptional domain located in the stem cell region (Galina et al., 2007; Mähönen et al., 2014). The highest level of PLT protein is found in the stem cell area, where it specifies stem cell identity (Aida et al., 2004; Galina et al., 2007; Mähönen et al., 2014). The progeny of the proximal stem cells express a lower level of PLT and undergo new rounds of rapid cell divisions before they start to elongate and differentiate (Galina et al., 2007; Mähönen et al., 2014). TACs require the expression of PLT genes, albeit to lower levels than in the stem cells (Galina et al., 2007; Mähönen et al., 2014).

Small RNAs are crucial regulators of gene expression in animals and plants and play a major role in development (Bologna and Voinnet, 2014). One class of small RNAs, the 21-nucleotide microRNAs (miRNAs), is defined by their biogenesis pathway, which requires the cleavage of a fold-back precursor RNA by a ribonuclease type III called DICERLIKE1 (Bologna and Voinnet, 2014). The miRNAs inhibit gene expression by forming a complex containing an ARGONAUTE (AGO) protein, generally AGO1 (Mallory et al., 2008), and then guiding the complex to specific target RNAs that are complementary to the miRNA. This represses

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the translation of the target RNAs or promotes their degradation, inhibiting production of the encoded protein.

The genome of Arabidopsis thaliana contains more than 200 miRNA genes grouped into families according to sequence similarity. The miR396 family is encoded by two genes, MIR396A and MIR396B, and regulates the expression of transcription factors belonging to the GROWTH-REGULATING FACTOR (GRF) class (Rodriguez et al., 2010; Debernardi et al., 2012). The GRF transcription factors are defined by the presence of the WRC and QLQ protein domains involved in DNA binding and protein-protein interactions, respectively (Kim et al., 2003). There are nine GRFs encoded in the Arabidopsis genome, and seven of them have a target site for miR396 (Jones-Rhoades and Bartel, 2004). The miR396-GRF interaction is conserved among angiosperms and gymnosperms (Jones-Rhoades and Bartel, 2004; Debernardi et al., 2012). It has been shown that overexpression of miR396 represses organ growth in Arabidopsis (Liu et al., 2009; Rodriguez et al., 2010; Bao et al., 2014; Liang et al., 2014b), whereas increased levels of the GRFs promote growth, especially in leaves (Kim et al., 2003; Horiguchi et al., 2005; Rodriguez et al., 2010), yet the mechanisms underlying the functions of the GRFs are largely unknown.

Here, we show that the miR396/GRF regulatory network regulates the transition of stem cells to transit-amplifying cells in the root meristem. GRFs are expressed in TACs, while miR396 is expressed in the SCN. The GRFs are essential for the function of the TACs: downregulation of their expression resulted in a decrease in the rate of the cell cycle and generated periclinal cell divisions typical of stem cells among the TACs. By contrast, the activity of miR396 is necessary to exclude the GRFs from the SCN. If unchecked, the GRFs induce the formation of distorted QC and columella cells. Corresponding with the phenotypic observations, high miR396 levels activate in the TACs the expression of PLT genes and other marker genes that are normally expressed in the SCN. In turn, PLT activity is required for the expression of MiR396 genes inside the SCN. Therefore, the interactions between miR396, GRF, and PLT initiate the transition between stem cells and the TACs.

RESULTS

miR396 Helps Determine the Architecture of the Root Meristem

Several GRF transcription factors are highly expressed in the meristematic region of the root (Supplemental Figure 1A), as determined using publicly available transcriptome data sets (Brady et al., 2007). We analyzed the expression pattern in more detail for two of these GRFs, GRF2 and GRF3, which are also regulated by miR396 (Figure 1A). To do this, we prepared translational reporters for GRF2 and GRF3, which consist of either GRF2 or GRF3 fused to GFP (GRF2-GRF and GRF3-GRF). Both GRF2 and GRF3 were detected in the meristematic zone (Figures 1B and 1C), and more specifically in TACs, consistent with a role of the GRFs in the promotion of cell proliferation.

We then examined the triple mutant grf1 grf2 grf3 and found the meristematic zone to be larger in size compared with the wild type (Figures 1D and 1H). Previous results have shown that a decrease of GRF levels due to the overexpression of miR396 causes a reduction in the number of cells and the size of the shoot apical meristem (Kim and Lee, 2006; Rodriguez et al., 2010). Therefore, although the increase in the root meristem size of grf1 grf2 grf3 was moderate, the result was unexpected, considering the previous data obtained using the aerial part of the plant. We generated a series of miR396 overexpressors to simultaneously downregulate all miR396-regulated GRFs (Figure 1E). We found that overall root growth was diminished in most of the transgenic plants expressing miR396 under the control of the strong 35S promoter (36 out of 50 primary transgenic plants) (Figure 1E; Supplemental Figure 2B). Cellular analysis of the line 35S:miR396 #2, which overexpresses moderate levels of miRNA MIR396b, showed that it had a larger meristematic zone due to an increase in both meristematic cell area and number (Figures 1F, 1I, and 1J). This is in agreement with the phenotype of grf1 grf2 grf3 and confirms that the miR396:GRF ratio has different effects in the root and shoot apical meristems. We used RT-qPCR to measure the expression of the GRFs in this transgenic line and in mutants and observed a significant reduction in the transcript levels of six of the GRF transcription factors (Supplemental Figure 2A). This may explain the stronger effects observed in this line compared with grf1 grf2 grf3 (Figures 1D and 1H). In addition, RT-qPCR analysis of the GRFs revealed that some of them were induced in this triple mutant (Supplemental Figure 1B), which might partially compensate for the loss of GRF1, GRF2, and GRF3.

Transgenic plants expressing the highest amounts of miR396, (35S:miR396 #1) had an even larger meristematic zone (Figure 1F). However, these plants also displayed other root defects, such as a reduced elongation zone (Figure 1F) and short mature cells (Supplemental Figure 2C). We conclude that the balance between miR396 and the GRFs has a primary effect on meristem size, whereas strong accumulation of miR396 severely affects the overall longitudinal patterning of the root.

To assess the importance of the miR396/GRF balance in the root meristem, we used an artificial miRNA that targets GRFs with higher efficiency than does miR396 by removing a bulged nucleotide between positions 7 and 8 of the binding site (Figure 1A) (Debernardi et al., 2012). Plants expressing this artificial miRNA also had an enlarged root meristem (Figure 1G) and exhibited stronger effects on root growth (Supplemental Figure 3) than miR396 overexpressors, consistent with the enhanced interaction with the GRFs.

Disruption of miR396 Function Reduces Root Meristem Size

As insertional knockouts in MIRNA precursors are difficult to obtain due to their small size, we turned to target mimicry technology, which is based on the expression of a noncoding RNA that sequesters the miRNA (Franco-Zorrilla et al., 2007; Todesco et al., 2010). We analyzed plants expressing a mimic directed against miR396 (MIM396) containing three miRNA binding sites designed as sponges for miR396 (Figure 2A). Analysis by laser scanning confocal microscopy of the root tip cellular architecture revealed that the decreased miR396 levels found in MIM396 plants (Supplemental Figure 4A) caused a moderate reduction in the size of the meristem (Figure 2B; Supplemental Figure 4B).
To determine the relevance of the repression by miR396 of specific GRFs, we analyzed the effect of silent mutations on the miR396 target site of GRF2 and GRF3. These mutations abolish the interaction with the miRNA, creating miRNA-resistant GRFs (rGRF2 and rGRF3) (Figure 2A). These GRF transgenes are transcribed from their own promoter regions but are insensitive to posttranscriptional repression by miR396. Both rGRF2 and rGRF3 plants accumulated higher levels of the corresponding GRF (Supplemental Figure 5A) and had defects in root growth (Supplemental Figures 5B and 5C). Analysis at the cellular level revealed a reduction in meristem size by rGRF2 and rGRF3 (Figures 2C and 2D) without any obvious effect on cell elongation (Supplemental Figures 5D and 5E).

To confirm that the downregulation of the GRFs was responsible for the meristem size increase of 35S:miR396 plants, we generated a dexamethasone (DEX)-inducible allele of rGRF3 expressed from its own promoter (rGRF3-GR). We introduced this construct into Arabidopsis plants and crossed them with plants harboring 35S:miR396. As expected, the resulting 35S:miR396 × rGRF3-GR plants have an enlarged meristem with respect to wild-type plants (Figure 2E). Treatment of 35S:miR396 × rGRF3-GR plants with DEX rescued the long-meristem phenotype (Figure 2F).

Figure 1. The miR396/GRF Balance Regulates Root Meristem Size and Growth.

In the micrographs, the white arrowheads mark the position of the QC, the yellow arrowheads mark the end of the meristem (Mz) where cells start to elongate, and the green arrowheads mark the end of the elongation zone (Ez).

(A) Depiction of a typical GRF gene. Note the miR396 target site (black box) and the interaction of GRFs with a mutant version of miR396 (miR396 7-8insG), which has a higher interaction energy. Also note the insertion of an additional nucleotide (highlighted in red) in miR396 7-8insG that eliminates the bulge present in the interaction between miR396 and the GRFs.

(B) and (C) Expression of GFP reporters of GRF2 (B) and GRF3 (C). GRF reporters are C-terminal translational fusions of GFP to the complete gene, including introns and their own promoter sequences. Bar = 50 μm.

(D) Root tip architecture 7 d after sowing of wild-type and grf1 grf2 grf3 triple mutant plants. Bar = 50 μm.

(E) Root growth phenotype 7 d after sowing of wild-type plants and three independent transgenic plants (35S:miR396) overexpressing increasing levels of miR396. Numerals above the photographs indicate the miR396 levels relative to wild-type roots as estimated by RT-qPCR. Bar = 1 cm.

(F) Root tip architecture in wild-type plants and plants from two representative transgenic lines expressing increasing amounts of miR396 (35S:miR396 #1 and #2, which accumulate 90- or 4-fold more miR396 compared with the wild type, respectively). Bar = 50 μm.

(G) Root tip architecture in plants from transgenic lines overexpressing miR396 7-8insG. This miRNA is an artificial miRNA created using the MIR319A precursor backbone. Bar = 50 μm.

(H) Number of cortex cells in the root meristem (Nm) of wild-type (Ws accession) and grf1 grf2 grf3 triple mutant plants. The asterisk indicates a significant difference from wild-type roots as determined by Student’s t test (P < 0.05). Ten cortex cell files from 10 plants of each genotype were scored for the number of meristematic cortex cells. The data shown are means ± se of 10 biological replicates.

(I) Number of cortex cells in the root meristem of wild-type (Col-0) and 35S:miR396 #2 plants. The asterisk indicates a significant difference from the wild type as determined by Student’s t test (P < 0.05). At least 10 meristematic cortex cells from 10 plants were measured. The data shown are means ± se of 100 individual cells.

(J) Length of mature cortex cells of wild-type (Col-0) and 35S:miR396 #2 plants. At least 10 mature cortex cells from 10 plants were measured. The data shown are means ± se of 100 individual cells.
confirming the importance of the miR396:GRF ratio in the control of root meristem size.

miR396 Modulates Cycling Cells

Despite their enlarged meristems, 35S:miR396 plants displayed a short-root phenotype, which might seem a contradiction at first sight. We hypothesized that the reduced levels of the GRFs were affecting the properties of the TACs. As a well-known characteristic of the TACs is a rapid division rate, we decided to analyze the duration of their cell cycle. An estimation of the average cell cycle duration of meristematic cortex cells (Ivanov and Dubrovsky, 1997) indicated an increase in 35S:miR396 and a reduction in rGRF3 compared with wild-type cells (Figure 3A).

We then used an experimental assay based on whole root cell cycle synchronization (Cools et al., 2010) to study the properties of the cell cycle in 35S:miR396 and rGRF3 plants. We measured the transcript levels of the mitotic CYCLINB1;2 and CYCLIN...
DEPENDENT KINASE B2:1 in the synchronized roots. Expression of these markers appeared earlier in rGRF3 with respect to wild-type roots (Figure 3B; Supplemental Figure 6). By contrast, the peak levels of these markers were both delayed and reduced in a 35S:miR396 background with respect to wild-type roots (Figure 3B; Supplemental Figure 6). Therefore, we propose that the GRFs not only affect the timely expression of mitotic cell cycle markers but also contribute to increasing their levels. These results indicate that the balance between miR396 and GRFs regulates the duration of the cell cycle in TACs, explaining
the overall reduction of root growth in 3SS:miR396 plants despite their enlarged meristems (Figure 1F and 1G). Furthermore, a delayed cell cycle probably explains the increase in cell size observed in the epidermis of 3SS:miR396 plants (Figure 1J).

Furthermore, expression of the G2-M-specific CYCLINB1;1 reporter within the TAC zone was located farther from the QC in 3SS:miR396 as compared with wild-type roots (Figures 3C and 3D), indicating that the miR396/GRF node affects functions in addition to the speed of the cell cycle, which might also be related to the establishment of the developmental zones along the longitudinal axis of the root.

miR396 Controls Periclinal Cell Divisions

Periclinal cell divisions of the stem cells can generate asymmetric daughters that produce different cell types, such as the epidermis (Ep)/lateral root cap (LRC) initials that generate the Ep and LRC cell layers. Once generated, these cells switch to anticlinal cell divisions, amplifying each of the cell types in the meristem before differentiation occurs (Campilho et al., 2006; Rost, 2011).

Typically, one or two cells are observed in wild-type plants with periclinal cell divisions, generating the Ep and LRC layers (Figures 4A and 4B; Supplemental Figure 7) (Campilho et al., 2006). Compared with the wild type, 3SS:miR396 plants harbored a significantly increased number of periclinal divisions that extended throughout the meristem (Figures 4C and 4D; Supplemental Figure 7A). As expected, an increase in the periclinal divisions occurred in grf1 grf2 grf3 mutants as well (Figures 4I and 4J; Supplemental Figure 7C).

The LRC/Ep marker WER-GFP confirmed a higher number of cell layers external to the cortex cells in 3SS:miR396 plants (Figures 4E and 4F). SOMBRERO (SMB), a root cap-specific NAC domain transcription factor, is expressed just after the asymmetric cell division that generates the root cap cells. It has been shown to prevent further generative divisions and promote cell maturation (Willemsen et al., 2008; Bennett et al., 2010; Fendrych et al., 2014). The analysis of an SMB-GFP reporter in 3SS:miR396 plants confirmed that the extra periclinal cell divisions generated extra LRC cells outside the Ep cell layer (Figures 4G and 4H), supporting a higher stem cell character of the distal portion of the meristem in these plants. Most of the ectopic periclinal divisions detected in 3SS:miR396 plants were associated with the enhanced meristem growth.
with the LRC/Ep cell layers; however, RT-qPCR experiments also showed an increase of CYCD6;1 levels in 35S:miR396 roots (Supplemental Figure 7D), which is necessary for the asymmetric cell division that generates cortex and endodermal cells (Sozzani et al., 2010; Cruz-Ramírez et al., 2012).

On the other hand, plants expressing rGRF3 and rGRF2 had a reduced number of LRC layers (Figures 4K and 4L; Supplemental Figure 8) and a low number of periclinal divisions associated with the Ep/LRC initials (Supplemental Figures 7A and 8). Accordingly, MIM396 plants also had fewer periclinal divisions (Supplemental Figure 7B), resembling the effect of rGRF3. Taken together, these results further support the importance of the miR396/GRF network in regulating the transition between stem cells and transit-amplifying cells.

miR396 Excludes the GRFs from the SCN

Further analysis of the stem cell region of rGRF3 plants revealed a distorted QC and columella cells displaying abnormal cell division patterns (Figure 5B). MIM396 plants also had a distorted QC (Figure 5C). Thus, repression of GRFs by miR396 is necessary for the homeostasis and function of the SCN. Consistent with this hypothesis, we found that MIR396a was strongly expressed in the QC and the columella (Figure 5D; Supplemental Figure 9A). We detected MIR396b at lower levels, which is consistent with small RNA sequencing data indicating that miR396a is the most abundant isoform in roots (Breakfield et al., 2012; Jeong et al., 2013). Still, we detected the expression of MIR396b in the

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Figure 5. Posttranscriptional Repression of the GRFs by miR396 in the SCN.

(A) to (C) SCN defects in rGRF3 (B) and MIM396 (C) in comparison with wild-type (A) plants. Bars = 50 µm.

(D) and (E) Expression patterns of MIR396A (D) and MIR396B (E) transcriptional GFP reporters. Bars = 50 µm.

(F) and (G) Region of miR396 activity detected with a sensor consisting of a nucleus-localized GFP without (F) (−) or with a miR396 binding site (G) (+). Bar = 50 µm.

(H) to (J) Expression pattern of GRF2 (H), GRF3 (I), and miR396-resistant GRF3 (rGRF3) (J) translational GFP reporters. Bars = 50 µm.
The miR396/GRF Regulatory Module Regulates the Gradient Distribution of PLT Genes.

(A) Expression of PLT1, PLT2, and BBM in 35S:miR396 and rGRF root tips as estimated by RT-qPCR. The data shown are means ± se of three biological replicates. Asterisks indicate significant differences from the wild type as determined by Student’s t test (P < 0.05).

(B) Expression of PLT1 and PLT2 after treatment with DEX (10 μM, 6 h) of plants transformed with an inducible rGRF3 (rGRF3-GR). The data shown are means ± se of three biological replicates. Asterisks indicate significant differences from the wild type as determined by Student’s t test (P < 0.05).

(C) to (H) Modified gradients of PLT1 and PLT2 in 35S:miR396 and rGRF3 roots. Bars = 50 μm.

(I) and (J) Quantification of the fluorescence intensity of proteins produced by PLT1-YFP (I) and PLT2-YFP (J) reporters in wild-type and 35S:miR396 roots. Purple bars indicate the QC region, while pink bars represent a region in the stele 50 μm above. The data shown are means ± se of eight biological replicates. Bars = 50 μm.

Downregulation of the GRFs Activates the Expression of Stem Cell Markers in TACs

AGAMOUS-LIKE42 (AGL42) is a MADS box transcription factor whose expression is enriched in the QC and adjacent stele and ground tissue stem cells (Nawy et al., 2005) (AGL42:GFP; Figure 6A). Overexpression of miR396 caused AGL42:GFP to be expressed in a broader area, consistent with a shootward expansion of the QC in plants having reduced GRF levels (Figure 6A).

To define the networks regulated by the miR396/GRF regulatory node, we performed transcriptome profiling of microdissected wild-type versus 35S:miR396 roots and discovered 600 induced and 135 repressed genes (P < 0.01 and fold change > 50%; Supplemental Table 1). Interestingly, a substantial number of genes that are coexpressed with AGL42 (Brady et al., 2007) were upregulated in the 35S:miR396 root samples (Figure 6B; Supplemental Table 2).

Next, we analyzed this data set for changes in the expression of genes important for the establishment of the QC. While there was no change in the expression of SCR or SHR, several PLT genes were upregulated in the 35S:miR396 transcript profiling data set (Figure 6C). Transcript levels for PLT1, PLT2, and BBM were increased in 35S:miR396 plants (Figure 7A; Supplemental Figure 12) but downregulated in plants expressing rGRF2 or rGRF3 (Figure 7A). Also, rGRF3-GR plants treated with DEX for 6 h showed a downregulation of PLT1 and PLT2 (Figure 7B).

PLT proteins have an expression maximum in the stem cell area, and their distribution forms a gradient along the longitudinal axis of the root (Figures 7D and 7G; Supplemental Figure 13A). This is instrumental for their function as dose-dependent regulators of root development (Galinha et al., 2007; Mähönen et al., 2014). The expression maxima of both PLT1-YFP and PLT2-YFP were reduced in the stem cell area by rGRF3, and their gradients faded away more abruptly compared with wild-type plants (Figures 7E and 7H; Supplemental Figures 13B and 13C). By contrast, both PLT genes were expressed at high levels in a broader region of 35S:miR396 roots (Figures 7C, 7F, 7I, and 7J; Supplemental Figure 13A).
levels present in the SCN are necessary for the activation of (Supplemental Figure 15). These results indicate that the high meristem (Mähönen et al., 2000), have more periclinal divisions in the

The expression of both GRFs Are Markers of Transit-Amplifying Cells in Roots

**DISCUSSION**

**GRFs Are Markers of Transit-Amplifying Cells in Roots**

The expression of both GRF2 and GRF3 was detected specifically in TACs. Neither GRF2 nor GRF3 was detected in the columella stem cells or in their daughters, the columella cells. As this tissue does not go through a transit-amplifying phase (Petricka et al., 2012), GRF expression seems to occur only in cells that undergo mitotic cycles to amplify the number of already established cell types.

GRF genes have regulatory sequences that are able to drive their transcription in a broader region, most conspicuously in the root SCN. This was shown through the introduction of mutations that prevent the binding of miR396 to GRF2 or GRF3 or by inactivating miR396 through target mimicry. Therefore, post-transcriptional repression by miR396 is essential to achieve the normal tissue-specific expression pattern of the GRFs.

In leaves, miR396 is highly expressed in expanding and differentiating cells and generates a basipetal gradient of expression of the GRFs, which coincides with proliferating cells (Rodriguez et al., 2010; Debernardi et al., 2012). Therefore, modifying GRF expression by miR396 seems to be a recurrent strategy to ensure that specific cells express these transcription factors. Interestingly, while MIR396a is the most highly expressed member of the miR396 family in leaves (Debernardi et al., 2012; Jeong et al., 2013; Liang et al., 2014a; Schommer et al., 2014), MIR396a is the most highly expressed in roots (Breakfield et al., 2012; Jeong et al., 2013; this work). MIR396b is induced in leaves by TCP4 (Schommer et al., 2014), a transcription factor involved in the repression of cell proliferation and the promotion of cell differentiation (Efroni et al., 2008; Schommer et al., 2014). In roots, we observed that MIR396 expression depends on PLT genes, indicating that miR396 is recurrently activated during development by different regulators.

**GRFs Repress Stem Cell-Like Properties in TACs**

The transition between stem cells and TACs in the Arabidopsis root is sharp, and the daughters of the stem cells immediately enter a defined pathway characterized by anticlinal cell divisions that amplify the established cell types. A decrease in GRF levels blurred this sharp transition: periclinal cell divisions, which are typical of stem cells, were observed among TACs, whereas markers of the QC and the stem cells extended their expression into the meristem. Cells in the SCN also proliferate at a much slower frequency than the surrounding transit-amplifying meristematic cells (Dolan et al., 1993; Kerk et al., 2000; Campilho et al., 2006; Cruz-Ramirez et al., 2013; Heyman et al., 2013), and a modification of the GRF levels in TACs modified the cell cycle length accordingly.

In addition, the peak of transit-amplifying cell divisions was shifted away from the stem cells, as has been reported recently for plants expressing high levels of PLT (Mähönen et al., 2014). Two pathways, directed by PLT and the SHR/SCR transcription factors, establish the QC and the stem cells (Petricka et al., 2012; Heyman et al., 2014). That only PLT transcripts responded to miR396 levels suggests that the GRFs specifically repress genes that establish stem cells.

It was recently shown that high auxin levels produce a narrow domain of PLT transcription from which PLT protein further spreads through growth dilution and cell-to-cell movement, generating a gradient of expression with a maxima in the SCN (Mähönen et al., 2014). In addition to these processes, our results show that downregulation of PLT levels in TACs is an active process that requires the activity of the GRFs. We propose that GRFs contribute to shape the gradient that defines the root
developmental zones. In this way, reduction in GRF activity causes a shootward expansion of the PLT gradient. The GRFs affected PLT transcript and protein levels. Because PLT protein is stabilized by secreted peptides (Matsuzaki et al., 2010; Zhou et al., 2010), we cannot rule out that the GRFs have an additional effect on the protein stability.

PLT1 has been shown to be repressed in the apical part of the embryo by HANABA TARANU and ANGUSTIFOLIA3 (AN3) (Kanei et al., 2012). AN3 has been also shown to interact with GRF transcription factors (Kim and Kende, 2004; Horiguchi et al., 2005; Debernardi et al., 2014), so we cannot dismiss the notion that the GRFs repress PLT genes in other tissues as well. However, our data show that the repression of PLT genes by the GRFs contributes to the quantitative regulation of PLT levels in the root meristem, as a reduction in the GRFs by miR396 or an increase in their levels by the use of rGRF transgenes or miR396 target mimics results in opposite changes of PLT expression levels. It is interesting that, in the most extreme cases, miR396 overexpression produces defects in cell elongation, which were also observed previously (Bao et al., 2014). Cell elongation is also inhibited by high local levels of PLT (Mählönen et al., 2014). Therefore, we propose that the interplay between miR396, the GRFs, and PLT transcription factors can also affect cell elongation in the most extreme cases.

The expansion of stem cell-like properties of the root meristem in plants with high levels of miR396 likely explains the decrease in the rate of the cell cycle and the increase of root meristem size. Therefore, we think that miR396 fulfills different functions in roots or the aerial part of the plant. Although we observed that miR396 is highly expressed in root stem cells and that its ectopic expression expands the domain of cells with stem cell-like properties, in leaves miR396 is associated with differentiating cells and its ectopic expression induces differentiation of the proliferating cells (Rodriguez et al., 2010; Debernardi et al., 2012). Still, in both tissues, the GRF transcription factors are expressed in rapidly dividing cells, which is ensured at least partially by the activity of miR396.

miR396-Mediated Exclusion of GRFs Is Essential for SCN Function

Reporters for both MIR396A and MIR396B promoters showed their highest expression in the SCN and columella cells. This activity of miR396 in the SCN was further validated by the use of a miR396 sensor and GRF reporter constructs. Each MIRNA was expressed in a specific subset of cells, suggesting that different signals contribute to their regulation, although the expression of both MIRNAs requires PLT activity.

Transgenes expressing miR396-resistant versions of GRF2 or GRF3 resulted in the expression of GRF transcription factors in the SCN, distorted QC, and columella cells. These results are consistent with a positive role of the GRFs in the promotion of the cell cycle. Roots with defects in miR396 activity, caused by the expression of either a miR396 target mimic or a miR396-resistant GRF, showed defects in the activity of the Ep/LRC initials, which is opposite to that seen in grf1 grf2 grf3 mutants or 35S:miR396. Exclusion of the GRFs seems to be necessary for periclinal cell divisions to occur, as can be seen in TACs with low GRF levels. A similar effect can be achieved by increasing PLT levels (Galinha et al., 2007). In turn, defects in the activity of the Ep/LRC initials and shorter meristems, as seen in rGRF3-expressing plants, are similar to the phenotypes observed in knockouts of PLT genes (Aida et al., 2004), further indicating that these transcription factors have opposite functions in the specification of stem cells and TACs.

The miR396/GRF network has been implicated in the developmental reprogramming processes unleashed upon biotic interactions of roots with cyst nematodes (Hewezi et al., 2012) or symbiotic rhizobia (Subramanian et al., 2008; Bazin et al., 2013). It would be interesting to determine if the mutual repression between GRFs and PLT described here also operates in the development of the specialized organs settled in each case.

The Regulatory Interaction between miR396, GRF, and PLT Controls the Transition of Stem Cells to Transit-Amplifying Cells

Taken together, our results indicate that the interactions between miR396, GRFs, and PLTs are required for the transition of stem cells into transit-amplifying cells. In the SCN, PLTs activate miR396, which excludes the GRFs. The GRFs become active in the transit-amplifying cells and dampen PLT expression (Figure 8G). In this way, miR396 establishes a molecular boundary to exclude GRFs from the SCN and set up a sharp transition from slowly proliferating cells with the capacity to generate different cell types to cells that amplify rapidly in number to ensure appropriate organ growth.

METHODS

Plant Materials, Growth Conditions, and Treatments

Arabidopsis thaliana accession Col-0 was used in most of the experiments. See Supplemental Table 4 for a list and a description of the transgenic lines and mutants used in this study. The miRNA target motif in GRFs was altered by introducing synonymous mutations into a wild-type GRF genomic fragment using the QuikChange Site Directed Mutagenesis Kit (Strategene).

Arabidopsis mutants plt1 plt2 (Galinha et al., 2007) and grf1 grf2 grf3 (Kim et al., 2003) are in the Wassilewskija (Ws) accession. The transgenic marker lines PLT1-YFP, PLT2-YFP (Galinha et al., 2007), WER-GFP (Lee and Schiefelbein, 1999), AGL42-GFP (Navy et al., 2005), and SMB-GFP (Fendrych et al., 2014) have been described previously.

Plants were grown in long photoperiods (16 h of light/8 h of dark) at 100 μmol photons m⁻² s⁻¹ at 21°C. For root analysis, plants were grown vertically on 1 × Murashige and Skoog salt mixture, 1% sucrose, and 2.3 mM MES, pH 5.8, in 1% agar. DEX (Sigma-Aldrich) was stored as 10 mM stocks in ethanol and used at the indicated concentrations (5 to 10 μM) for the indicated periods (6 to 72 h). The root meristem was synchronized in the G1/S transition using hydroxyurea as described (Cools et al., 2010), and the expression of cell cycle markers was followed by RT-qPCR.

Expression Analysis

Total RNA was isolated from root tissue using Tripure isolation reagent (Roche). Total RNA (0.5 μg) was treated with RNase-free DNase (Promega). Then, first-strand cDNA synthesis was performed using SuperScript III Reverse Transcriptase (Invitrogen). PCR was performed in a Mastercycler ep realplex thermal cycler (Eppendorf) using SYBR Green I
(Roche) to monitor double-stranded DNA synthesis. qPCR for each gene was done on at least three biological replicates with technical duplicates for each biological replicate. The relative transcript level was determined for each sample, normalized to the PROTEIN PHOSPHATASE2A CDNA level (Czechowski et al., 2005). Mature miR396 levels were determined by stem-loop RT-qPCR as described previously (Debeirni et al., 2012). Primer sequences are given in Supplemental Table 3. To visualize GUS reporter activity, roots of transgenic plants were subjected to GUS staining, as described previously (Donnelly et al., 1999).

**Microarray Analyses**

Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) from microdissected meristems of wild-type and 35S:miR396 roots grown on vertical square plates (10 × 10 × 2 cm) for 7 d. Microarray analyses using the Affymetrix ATH1 platform were performed on three biological replicates as described (Schmid et al., 2005). Normalized expression estimates were obtained using Guanine-Cytosine Robust Multi-Array Average (GCRMA) as described (Schmid et al., 2005). Normalized expression estimates were calculated for each individual root using the following equation: T = (ln2 Nm Le) V⁻¹, where Nm is the number of meristematic cells in one file of the cortex, Le is the length of fully elongated cortex cells in μm, and V is the root growth rate calculated as μm/h (Ivanov and Dubrovsky, 1997).

**Accession Numbers**

Accession numbers (Arabidopsis Genome Initiative locus identifiers) for the genes described here are provided in Supplemental Table 3. Microarray data have been deposited in the Gene Expression Omnibus database (GSE58807).

**Supplemental Data**

**Supplemental Figure 1.** GRFs in Root Development.

**Supplemental Figure 2.** Molecular and Cellular Characterization of 35S:miR396.

**Supplemental Figure 3.** Overexpression of a Hyperactive miR396 Variant Causes Stronger Root Phenotypes.

**Supplemental Figure 4.** Decreased Meristem Size in MIM396 Plants.

**Supplemental Figure 5.** Characterization of rGRF2 and rGRF3 Plants.

**Supplemental Figure 6.** Control of Cycling Cells by miR396.

**Supplemental Figure 7.** Number of Periclinal Cell Divisions in Epl/LRC Initials of 35S:miR396, rGRF3, MIM396, and grft1 grf2 grf3 Plants.

**Supplemental Figure 8.** rGRF2 Perturbs the Stem Cell Niche and Modifies the Pattern of Periclinal Divisions.

**Supplemental Figure 9.** Expression Pattern of MIR396 Genes.

**Supplemental Figure 10.** A miR396 Sensor Is Excluded from the Stem Cell Niche.

**Supplemental Figure 11.** Posttranscriptional Regulation of GRF2 by miR396.

**Supplemental Figure 12.** Quantitative Regulation of PLT by the miR396/GRF Network.

**Supplemental Figure 13.** Regulation of PLT Expression Gradient by the miR396/GRF Network.

**Supplemental Figure 14.** PLT Activity Is Necessary for MIR396 Expression in the SCN.

**Supplemental Figure 15.** PLT Induces miR396 to Exclude GRFs from the SCN.

**Supplemental Table 1.** Genes Differentially Expressed in 35S:miR396 Root Meristems as Compared with the Wild Type.

**Supplemental Table 2.** Expression of AGL42 Coexpressed Genes in 35S:miR396 Root Meristems as Compared with the Wild Type.

**Supplemental Table 3.** Locus IDs and Oligonucleotide Primers Used in RT-qPCR.

**Supplemental Table 4.** Binary Plasmids Used in This Study to Generate Transgenic Lines.

**ACKNOWLEDGMENTS**

We thank Ben Scheres for the plt1 plt2 mutants and the PLT1:PLT1-YFP and PLT2:PLT2-YFP reporters; Jen Hoe Kim for the grf1 grf2 grf3 mutant; Moritz Nowack for the SMB-GFP reporter; Alexis Maizel, Renze Heidstra, Ben Scheres, and members of the Benfey and Palatnik laboratory for advice and reading of the article; and Rodrigo Vena, Enrique Morales, and Lia Pietrasanta for help with microscope imaging. M.F.E., J.M.D., and M.A.M. were supported by fellowships from CONICET. M.F.E. was also supported by the Josefinap Frants Foundation, T.C. is a Postdoctoral Fellow of the Research Foundation-Flanders. R.E.R. and J.F.P. are members of CONICET. P.N.B.’s laboratory was supported by the National Institutes of Health (Grant R01-GM043778) and by the Gordon and Betty Moore Foundation (through Grant GBMF3405). R.E.R. was supported by ANPCyT (Grants PICT2010/1847 and PICT2012/1686). The majority of studies were supported by grants to J.F.P. (ANPCyT and Howard Hughes Medical Institute).

**AUTHOR CONTRIBUTIONS**


Received May 22, 2015; revised September 30, 2015; accepted November 11, 2015; published December 8, 2015.

**REFERENCES**


MicroRNA miR396 Regulates the Switch between Stem Cells and Transit-Amplifying Cells in Arabidopsis Roots
Ramiro E. Rodriguez, María Florencia Ercoli, Juan Manuel Debernardi, Natalie W. Breakfield, Martin A. Mecchia, Martin Sabatini, Toon Cools, Lieven De Veylder, Philip N. Benfey and Javier F. Palatnik

Plant Cell; originally published online December 8, 2015;
DOI 10.1105/tpc.15.00452

This information is current as of October 20, 2017

Supplemental Data /content/suppl/2015/11/13/tpc.15.00452.DC1.html
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