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Ercoli, Maria Florencia

Rojas, Arantxa

Debernardi, Juan

et al.

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SHORT COMMUNICATION

Control of cell proliferation and elongation by miR396

María Florencia Ercoli, Arantxa M. L. Rojas, Juan Manuel Debernardi, Javier F. Palatnik, and Ramiro E. Rodriguez

Instituto de Biología Molecular y Celular de Rosario (IBR), CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

ABSTRACT

The combinatory effects of cell proliferation and cell elongation determines the rate at which organs growth. In the root meristematic zone cells both divide and expand, while post-mitotic cells in the elongation zone only expands until they reach their final size. The transcription factors of the *GROWTH-REGULATING FACTOR* (*GRF*) class promote cell proliferation in various plant organs. Their expression is restricted to cells with a high proliferative capacity, yet strong downregulation of the GRF activity compromise the plant survival. Part of expression pattern of the *GRFs* is ensured by the post-transcriptional repression mediated by the conserved microRNA miR396. Here we show the quantitative effects in root growth caused by *GRF* depletion in a series of transgenic lines with different miR396 levels. We show that high miRNA levels affect cell elongation and proliferation in roots. Detailed analysis suggests that cell proliferation is restricted due to a reduction in cell cycle speed that might result from defects in the accumulation of mitotic cyclins. The results provide insights into the participation of the miRNA-GRF regulatory network in root development.

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The indeterminate growth of roots is sustained via coordinated cell division and elongation at the root tip. This region is organized in developmental zones distributed consecutively in the longitudinal axis, each devoted to a particular developmental process (Fig. 1A). Closest to the tip, the stem cell niche generates different cell types from common initials by asymmetric formative cell divisions. Above this region, each cell type proliferates in the meristematic zone (MZ) at a much higher speed to amplify cell number. Later, cells cease to proliferate and start to expand and further differentiate in the elongation (EZ) and maturation zones.[1]

The *GROWTH-REGULATING FACTOR* is a plant specific family of transcription factors defined by the presence of the WRC and QLQ protein domains.[2-4] Analysis of *GRF* mutants and overexpressors have shown that these transcription factors promote cell proliferation during leaf development.[3,5,6] Seven out of the 9 Arabidopsis *GRFs* have a binding site for microRNA (miRNA) miR396.[7] The post-transcriptional repression of *GRFs* by miR396 results in the co-expression of the transcription factors with proliferating leaf cells.[8,9] Accordingly, miR396 overexpression causes a significant reduction of *GRF* expression and smaller leaves with reduced cell number.[8-10]

GRFs have also been implicated in the development and growth of other plant organs and structures.[11] For example, *GRFs* are required for the development and maintenance of the shoot apical meristem (SAM), as strong miR396 overexpressors in sensitized backgrounds[9] or quadruple *grf1 grf2 grf3 grf4* mutants[12] lacked a functional SAM. Also, plants with severe reductions in *GRF* expression due to overexpression of miR396 had defects in flower development, including reduced numbers of organs and missing carpels, that compromise plant fertility.[13]

It has recently been shown that *GRFs* are expressed in the MZ of roots (Fig. 1A) but excluded from the stem cells through repression by miR396.[4] A mild reduction in *GRF* levels achieved by miR396 overexpression affects root growth by reducing the activity of the meristem,[4] further confirming the role of the *GRFs* in cell proliferation. However, a quantitative analysis of the importance of *GRF* activity in root development is still missing, in part due to the inabilities to maintain stable lines with severely reduced GRF levels, as mentioned before. Therefore, to better characterize the effect on root growth caused by higher reductions in *GRFs* levels we analyzed a population of independent primary transgenic (T1) plants expressing different levels of miR396.

We analyzed the root growth of 34 independent transgenic plants harboring a *35S:miR396a* construct and 21 control plants transformed with an empty vector. We observed that all the *35S:miR396a* plants displayed an inhibition of the root growth when compared to plants transformed with an empty vector, albeit the degree of inhibition varied for the different lines as expected. Next, we pooled the roots from this population in 2 groups according to the magnitude of the root growth inhibition and estimated the levels of mature miR396 by RT-qPCR. We found a correlation between miR396 overexpression and root growth inhibition (Fig. 1B–C), confirming a correlation between miRNA levels and the defects in the organ elongation.

CONTACT Ramiro E. Rodriguez rrodriguez@ibr-conicet.gov.ar
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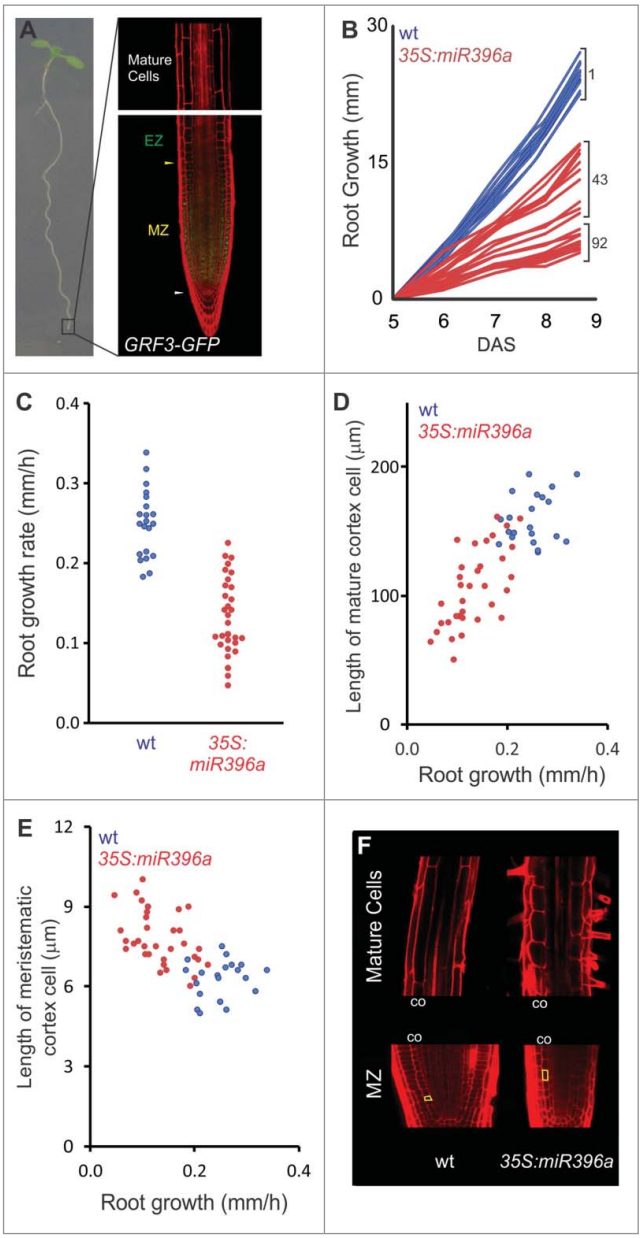

Figure 1. MiR396 controls the size of both mature and meristematic cells. (A) Root tip architecture and expression of a *GRF3-GFP* reporter. The white arrow head marks the distal border of the meristematic zone (MZ). The yellow arrow head marks the end of the meristem where cells start to expand in the elongation zone (EZ). (B). Distribution of root growth phenotypes in a population of primary transgenic plants transformed with an empty vector (wt) or a vector over-expressing miR396a under the 35S promoter (*35S:miR396a*). Numerals next to graphs indicate the mature miR396 levels relative to wild-type for the corresponding pool of roots (indicated with a bracket). (C) Root elongation speed (mm/h) of each wild-type and *35S:miR396a* plant in the T1 population analyzed. (D) Length of mature cortex cells in wild-type and *35S:miR396a* plants. (E) Length of meristematic cortex cells in wild-type and *35S:miR396a* plants. (F) Detailed view of mature (top) and meristematic (bottom) cells of wild-type and *35S:miR396a* plants. A particular cortex cell in the meristematic region is highlighted in yellow. co, cortex cell file.

Actually, the strongest miR396 overexpressors have a 70% reduction of the root elongation rate confirming the importance of the miRNA and the GRFs in root growth.

To better understand at the cellular level the causes of this phenotype we analyzed Propidium Iodide stained roots with Laser Scanning Confocal Microscopy. This allows for the detailed analysis of the cellular driving forces that contribute to organ growth, namely, cell proliferation in the MZ and cell elongation in the EZ (Fig. 1A).

First, we focused on the effects of miR396 on cell expansion and determined the size of the mature cortical cells for the 34 *35S:miR396a* transgenic plants and the 21 control plants. We found that plants with the highest levels of miR396 have a 50% reduction in the length of mature cortical cells (Fig. 1F). There was a good correlation between the reduction in mature cell size and the inhibition in root growth rate in the different *35S:miR396a* plants (Fig. 1D). Therefore, at least part of the short-root phenotype of *35S:miR396a* plants can be explained by a defect in cell elongation, as mature cortex cells of plants with reduced levels of *GRFs* were shorter when compared to wild-type.

Then, we analyzed the cell size on actively proliferating cells. We found that plants overexpressing miR396 have a significant increase in the length of meristematic cortex cells (Fig. 1E, F). This indicate that miR396 overexpression have opposing effects in the meristematic cells when compared to the mature cells, as it promotes elongation of dividing cells but diminishes the size of mature cells (Fig. 1F).

Next, we studied the production of new cells in the meristem as there is usually a correlation between root growth and cell proliferation in this region.[14] We found that plants with the highest levels of miR396 have a 40% reduction in the rate of generation of new cortex cells. Therefore, a defect in the ability of these meristems to produce new cells correlated with the inhibition of root growth in miR396 overexpressors (Fig. 2A). We found that this effect was caused by a slower cell cycle, as the average cell cycle time for cortex cells in the meristem of *35S:miR396a* plants was at least doubled in the most affected plants (Fig. 2B).

The mitotic cell cycle is a tightly controlled process organized in consecutives stages. In the S-phase, the cell replicates its genome while in the M phase the duplicated genomes are distributed between the two daughter cells. Both phases are preceded by preparative gap phases, G1 and G2, and the transition between them is regulated by the oscillating activity of various cell cycle regulators, including as key components the complexes between cyclins (CYC) and cyclin dependent kinases (CDK).[15]

To explore how miR396 overexpression affects the cell cycle we analyzed the expression of cell cycle markers[16] in transcriptome datasets from micro-dissected meristems of wild-type versus *35S:miR396a* roots.[4] We found that the expression of S-phase genes[16] was reduced in *35S:miR396a* (Fig. 2C and Table S1), indicating that the population of cells undergoing the mitotic cycle were depleted from cells in this particular stage of the cell cycle. When Mitosis Specific Genes (MSG)[16] were analyzed, we found that some genes were enriched, while others were depleted (Fig. 2D and Table S1), suggesting that meristematic cells might be delayed at a certain intermediate stage of the G2 to M transition.

The transitions between the phases of the cell cycle are driven by the oscillating expression profiles of cell cycle regulators that result from the combination of complex transcriptional, post-transcriptional and post-translational mechanisms.[15,17,18] Among the later ones, protein degradation via the ubiquitin-proteasome pathway is responsible for

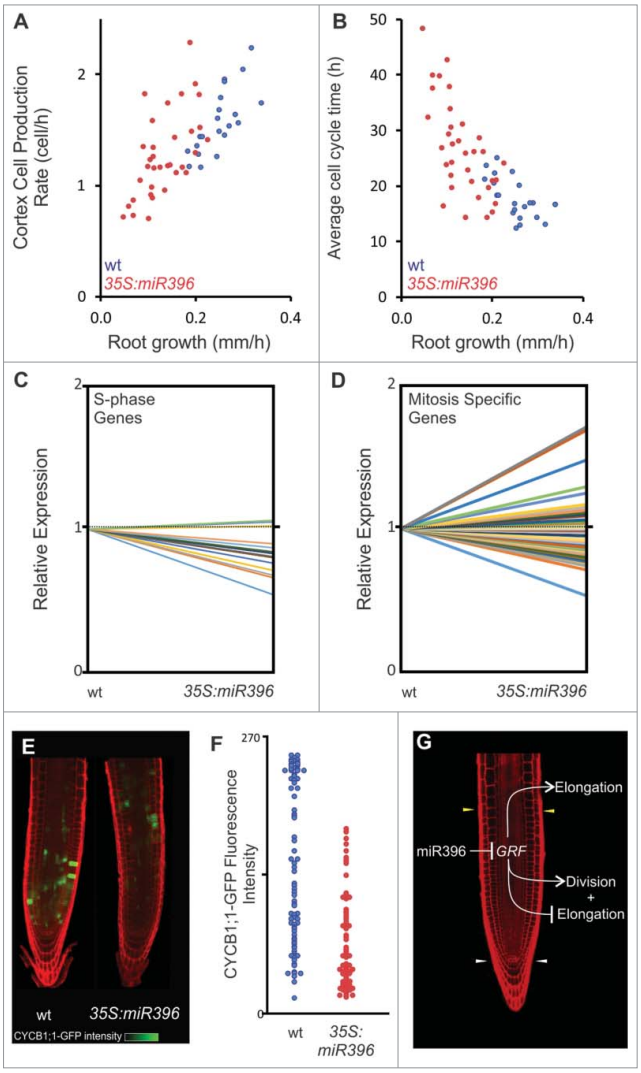

Figure 2. MiR396 controls the activity of the root meristem. (A) Cortex cell production rate by the root meristem (cell/h) in wild-type and *35S:miR396a* plants. (B) Average duration of the cell division cycle in meristematic cortex cells from wild-type and *35S:miR396a* plants. (C-D) Expression of S-phase (C) and Mitosis Specific Genes (D) in microdissected root apical meristems of *35S:miR396a* roots as estimated with ATH1 microarrays. (E) Defects in the accumulation of the mitotic marker *CYCB1-1GFP* in wild-type and *35S:miR396a* root apical meristems. (F) Fluorescence intensity of cortex cells expressing the *CYCB1;1:GFP* reporter in wild-type and *35S:miR396* root apical meristems. At least 70 cells from 20 plants were analyzed for each genotype. (G) Model summarizing the contributions of the miR396/*GRF* regulatory module in the control of cell proliferation and elongation.

clearing cell cycle regulators. To determine if miR396 overexpression affected the accumulation of mitotic cyclins at the protein level we analyzed quantitatively the expression of a *CYCB1-1GFP* reporter in wild-type and *35S:miR396a* plants. We found that the maximum fluorescence intensity was reduced 30% in *35S:miR396a* plants with respect to wild type plants (Fig. 2E, F). Therefore, cell cycle regulators seem to fail to accumulate in *GRF*-deficient plants, which might explain the delay observed in the cell cycle.

Our results show that miR396 overexpression, and hence GRF depletion, affects negatively 2 of the main driving forces that determine root organ growth, *i.e.* cell elongation and cell cycle speed (Fig. 2G). The *GRFs* are highly expressed in the MZ, where a link with cell proliferation can be easily explained. However, they are not only necessary to promote cell proliferation, but also needed for cell elongation outside the meristem as mature root cortex cells are significantly reduced in size in *35S:miR396* plants. The mechanisms by which the GRFs control cell expansion, both inside and outside the meristem, are currently unknown and future work will be required to understand the mechanisms and biological implications of these effects.

Materials and methods

Plant materials, growth conditions, and treatments

Arabidopsis thaliana accession Col-0 was used in all the experiments. The phenotypic characterization of the transgenic population of miR396a overexpressing plants was performed using plants transformed with a vector containing the *MIR396A* precursor under the *35S* promoter (*35S:miR396a*)[4] or the empty vector as control. 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 1X Murashige and Skoog salt mixture, 1% sucrose, and 2.3 mM MES, pH 5.8, in 1% agar.

Gene expression analyses

Total RNA was isolated from root tips using Tripure RNA isolation reagent (Roche). Total RNA (0.5 μg) was treated with RQ1 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. Mature miR396 levels were determined by stemloop RT-qPCR as described previously.[8] qPCR was done on at least 3 biological replicates with technical duplicates for each biological replicate.

Microscopy

Roots were stained with 10 mg/mL propidium iodide for 1 min and mounted in water. Laser confocal scanning microscopy was performed with a 20X, 0.75-NA lens on a Nikon Eclipse TE-2000-E microscope equipped with a C1-si confocal scanning head, using the 488-nm laser line for excitation, a 515/30-nm band-pass filter for GFP, and a 605/75-nm band-pass filter for propidium iodide detection. Cellular parameters and fluorescence intensities were analyzed with Fiji.[19]

The average length of meristematic cortex cells was estimated by determining the average cell size from the 5th to the 15th cell from the QC.[4] The average rate of cortex cell production by the meristem was estimated as V/Le, where V is the root growth rate calculated in μm/h while Le is the length of fully elongated cortex cells in μm.[20] The average duration of the cell cycle for meristematic cortex cells was calculated for each individual root using the following equation: $T = (\ln 2\ Nm\ Le)\ V^{-1}$, 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.[21]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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