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Repression of Cell Proliferation by miR319-Regulated TCP4

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ABSTRACT **Leaf development has been extensively studied on a genetic level. However, little is known about the interplay between the developmental regulators and the cell cycle machinery—a link that ultimately affects leaf form and size. miR319 is a conserved microRNA that regulates TCP transcription factors involved in multiple developmental pathways, including leaf development and senescence, organ curvature, and hormone biosynthesis and signaling. Here, we analyze the participation of TCP4 in the control of cell proliferation. A small increase in TCP4 activity has an immediate impact on leaf cell number, by significantly reducing cell proliferation. Plants with high TCP4 levels have a strong reduction in the expression of genes known to be active in G2-M phase of the cell cycle. Part of these effects is mediated by induction of miR396, which represses Growth-Regulating Factor (GRF) transcription factors. Detailed analysis revealed TCP4 to be a direct regulator of *MIR396b*. However, we found that TCP4 can control cell proliferation through additional pathways, and we identified a direct connection between TCP4 and *ICK1/KRP1*, a gene involved in the progression of the cell cycle. Our results show that TCP4 can activate different pathways that repress cell proliferation.**

Key words: microRNA; miR396; miR319; TCP; GRF; ICK1; KRP1; proliferation.

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INTRODUCTION

The development of multicellular organisms ultimately relies on a precise control of cell proliferation and expansion. The mechanisms controlling the progression through the cell cycle are largely conserved across plant and animal kingdoms (Gutierrez, 2009; Inze and De Veylder, 2006). It is, however, the integration of these core pathways composed of cyclins and cyclin-dependent kinases with the specific developmental program of an organism which will generate a diversity of organ sizes and shapes—a process much less understood. Leaves are organs generated at the flanks of the plant shoot apical meristem, first appearing as rod-shaped primordia, which then expand and grow to form flat laminas as seen in *Arabidopsis thaliana*. Cell division occurs first throughout the small developing leaf primordia, then becomes restricted to the proximal part of the organ, until it ceases rather abruptly (Donnelly et al., 1999; Beemster et al., 2005; Kazama et al., 2010; Andriankaja et al., 2012; Powell and Lenhard, 2012; Rodriguez et al., 2014). Dispersed meristematic cells will continue to proliferate for a longer time, producing stomata and vascular cells, until division stops overall (White, 2006). Afterwards, the organ grows by cell enlargement. Many genes have been identified as regulators of leaf development in *Arabidopsis*, although the mechanistic connections between the different pathways remain mostly unknown (Gonzalez et al., 2012; Rodriguez et al., 2014).

The TCPs are a known class of transcription factors that control many aspects of plant growth and especially leaf development (reviewed in Martin-Trillo and Cubas, 2010). They are a plant-specific group of transcription factors that resemble the ubiquitous bHLH class, and 24 of them have been identified in *A. thaliana*. According to the sequence of the TCP domain that is responsible for DNA binding and protein–protein interaction, they can be divided in two subgroups: classes I and II (Kosugi and Ohashi, 2002). In general, class I TCPs have been associated with the promotion of the cell cycle machinery whereas class II TCPs have been suggested to promote

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the arrest of the cell cycle (Kosugi and Ohashi, 2002; Li et al., 2005).

Five class II TCPs (*TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24*) and their homologs in different species are regulated by the conserved microRNA miR319. High levels of miR319 and/or inactivation of miR319-regulated *TCP*s cause important changes in *Arabidopsis* leaf morphogenesis and the generation of crinkled leaves (Palatnik et al., 2003; Koyama et al., 2007; Efroni et al., 2008). Seminal work in snapdragon has implicated the *TCP*s in the coordination of cell proliferation, which in turn is necessary to generate a flat leaf lamina. Additionally, miR319-regulated TCPs have been described to control leaf development (Nath et al., 2003; Palatnik et al., 2003; Koyama et al., 2007; Schommer et al., 2008; Koyama et al., 2010; Sarojam et al., 2010), cell differentiation (Sarojam et al., 2010; Sarvepalli and Nath, 2011), biosynthesis and/or signaling of several hormones (Schommer et al., 2008; Koyama et al., 2010; Yanai et al., 2011; Efroni et al., 2013), and also flowering time and flower development (Palatnik et al., 2003; Crawford et al., 2004; Schommer et al., 2008; Rubio-Somoza and Weigel, 2013). Furthermore, TCP proteins interact with clock proteins (Giraud et al., 2010) and chromatin remodelers (Efroni et al., 2013). The functions of miR319-regulated *TCP*s have also been described in different plants with simple and compound leaves such as *Antirrhinum majus* (Nath et al., 2003; Crawford et al., 2004) and *Solanaceae* species (Ori et al., 2007; Shleizer-Burko et al., 2011; Yanai et al., 2011).

Previous work revealed a heterochromatic function of these transcription factors in leaf development (Efroni et al., 2008). Leaves overexpressing miR319 have a lower differentiation index compared to wild-type, implying a slower and delayed maturation of the organ due to a reduction in TCP activity (Efroni et al., 2008). In contrast, plants with higher TCP activity have an early onset of maturation and a decrease of overall cell division (Efroni et al., 2008; Sarvepalli and Nath, 2011).

Here, we characterize the role of TCP4 in the repression of cell proliferation in *A. thaliana* leaves. Already slight up-regulation of TCP4 activity has a primary effect on the expression of cycling genes and the number of cells in leaves. We found that the miR319-regulated TCPs are able to directly regulate different pathways that can inhibit cell proliferation. We found that TCP4 directly activates *MIR396b*, which encodes a miRNA that represses cell proliferation. Furthermore, we describe a newly characterized target for TCP4, the *CYCLIN-DEPENDENT KINASE INHIBITOR 1* (*ICK1*)/*KIP RELATED PROTEIN1* (*KRP1*), which might provide a link between the miR319-regulated TCPs and core genes involved in the progression of the cell cycle. The repression of cell proliferation by TCP4 might be a causal mechanism activating certain differentiation programs of the leaf.

RESULTS AND DISCUSSION

Modification of Leaf Shape and Cell Number by Small Increases of TCP4 Levels

An EMS suppressor screen performed on the *jaw*-D mutant that overexpresses miR319a identified *TCP4* alleles with point mutations in the miR319-binding site and were called *soj* (suppressor of *jaw*-D) (Palatnik et al., 2007). *soj8* mutants have rosettes without the typical crinkly feature of the jaw-D mutant leaf and are also of smaller size than wild-type (Palatnik et al., 2007; Rodriguez et al., 2010) (Figure 1A). Apart from these plants, we also analyzed the T1 generation of transgenic plants expressing wild-type *TCP4* from the 35S promoter. Compared to a control population of plants transformed with an empty vector we found smaller plants (eight out of 25 primary transgenic plants) similar to *soj8* (Figure 1A). That both *soj8* and *35S:TCP4* plants had similar phenotypes suggested that an increase in TCP levels and/or activity reduced leaf size and that miR319 likely regulates TCP4 levels in *Arabidopsis* quantitatively.

Transgenic plants carrying a *TCP4* transgene expressed from its own promoter (using a 2.3-kb fragment upstream of the coding sequence) fused to *GFP* and containing synonymous mutations in the miR319-binding site (*rTCP4*, Figure 1B and 1C) also cause a reduction in the size of the leaves (Schommer et al., 2008), further confirming the results obtained with *soj8* and wtTCP4. That *rTCP4–GFP* caused stronger reduction of leaf size than *soj8* (Figure 1A and 1B) is not surprising, since *soj8* harbors a single point mutation in the miR319-binding site, while seven mutations were introduced in the *rTCP4–GFP* transgene (Figure 1C). We determined *TCP4* transcript levels in *soj8* and *rTCP4–GFP* plants and observed two-fold and four-fold increases with respect to wild-type plants (Figure 1D). These results are in agreement with stronger reduction of miR319 regulation in *rTCP4–GFP* with respect to *soj8* and wild-type plants. While analyzing the different primary transgenic plants, we noticed that small increases in TCP4 levels caused only a reduction in leaf size, while a stronger increase had more general effects modifying the leaf phyllotaxis (Figure 1B); a similar effect was noticed for *35S:TCP4* (not shown).

Next, we outcrossed the *jaw*-D allele from the *soj8* mutant and observed leaf size and shape. In wild-type background, the *soj8* mutation manifests with smaller leaves with a lanceolated blade compared to Columbia wild-type plants (Figure 1E). To describe the leaf form better, we measured as an example the angle with which the leaf blade starts from the petiole in the fourth leaf and determined that, in *soj8*, this angle was 152° compared to 124° in wild-type (Figure 1E). The margins of the *soj8* leaves were also smoother than wild-type—an effect that was especially evident in the later-occurring adult leaves, showing that the regulation of *TCP4* by miR319 is important to generate the typical leaf serrations of wild-type plants.

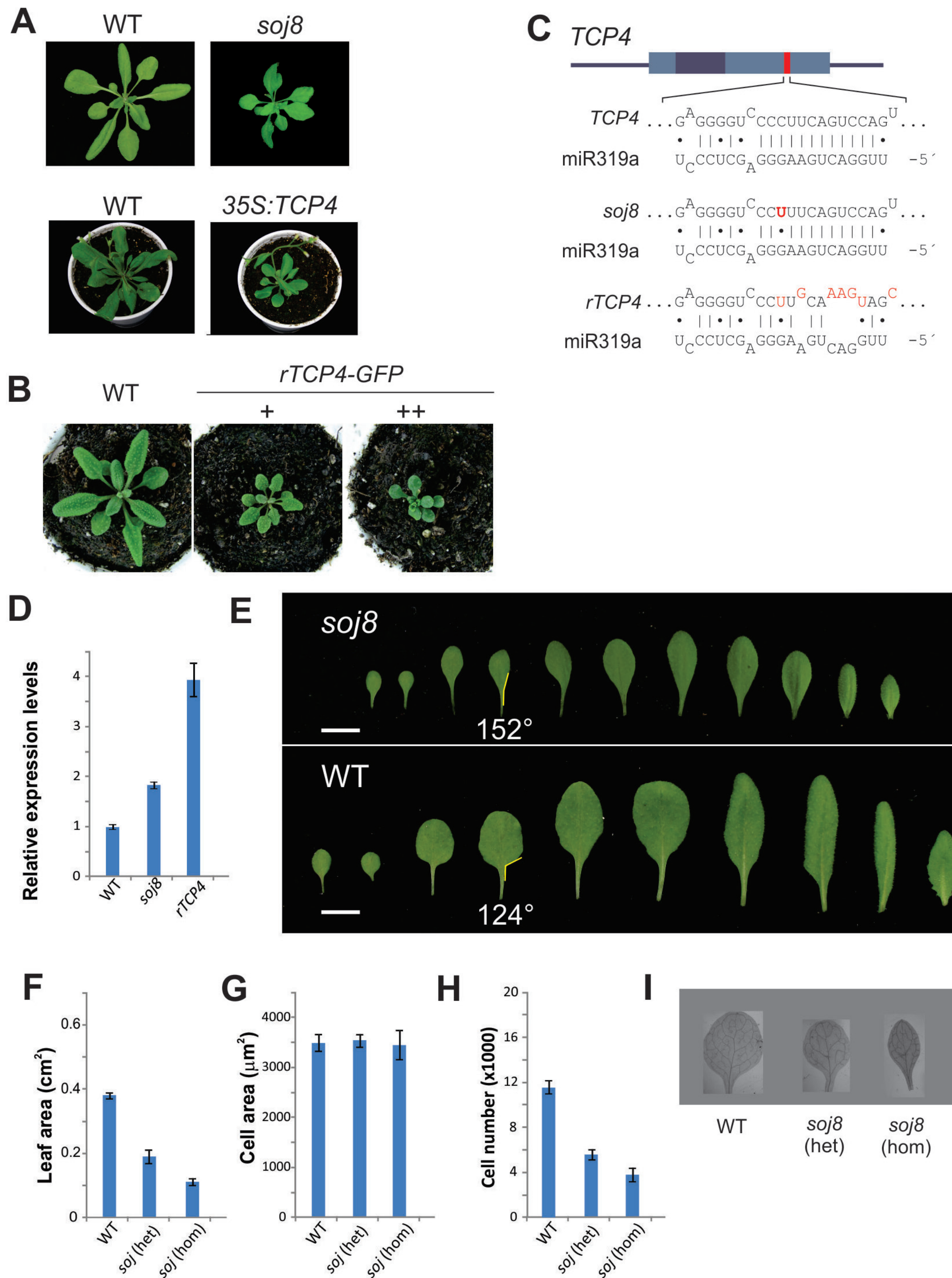

Figure 1 Effect of Increased TCP4 Levels on Leaf Size and Shape.

(A) Four-week-old rosettes of *Arabidopsis* lines with increased *TCP* levels (*soj8*, *35S:TCP4*) compared to control plants (transformed with an empty-vector construct), having smaller rosettes.

(B) Four-week-old rosettes of two representative *rTCP4–GFP* lines with stronger reduction in rosette size compared to control plants transformed with empty vector.

(C) Scheme demonstrating the interaction of TCP4 with microRNA319 in wild-type (WT), *soj8*, and *rTCP4*. Bases that differ from WT are indicated in red.

(D) Relative *TCP4* transcript levels measured in apices of *soj8* and *rTCP4* lines, compared to WT.

As we were mainly interested in understanding the effect of TCP misregulation on leaf size, we measured the cell size and cell number of mesodermal parenchyma cells in fully expanded first leaves of *soj8* plants without carrying the *jaw*-D allele. We found that the reduction in leaf size is due to a reduction in the number of cells (Figure 1F–1I), not a reduction in cell size. Furthermore, we observed that the effect of the *soj8* allele on cell number is quantitative because heterozygous soj8/+ plants have an intermediate phenotype between wild-type and homozygous *soj8* mutants (Figure 1F–1I). Taken together, these results show that a small increase in TCP4 levels and/or activity causes a strong effect on the number of cells and the shape of the margins of *Arabidopsis* leaves.

Repression of Mitosis-Specific Genes by TCP4

To bring insights into the regulation of cell proliferation by miR319-regulated *TCP* transcription factors, we analyzed microarray experiments (ATH1 Affymetrix) of wild-type plants and transgenic plants carrying an *rTCP4* transgene, which displayed a similar phenotype to *soj8* (Figure 1A and 1B) (Palatnik et al., 2003; Schommer et al., 2008). To identify the effect of this genotype on genes that are of importance for cell proliferation, we made use of a group of genes that are known to peak during the G2-M phase of the cell cycle in *Arabidopsis* suspension cultures (80 of these genes are present in ATH1 arrays) (Menges et al., 2003). We found that high TCP4 activity caused a clear repression of those known mitotic cell cycle genes (Figure 2A).

We further analyzed the *rTCP4* arrays, which showed down-regulation of 1356 genes compared to wild-type (fold change > 2, $p < 0.05$) (Supplemental Table 1). We examined Gene Ontology (GO) terms for enrichment among these genes (Brady et al., 2007) and found that five out of the top six categories were related to cell division and the progression of the cell cycle (Figure 2B). Next, we

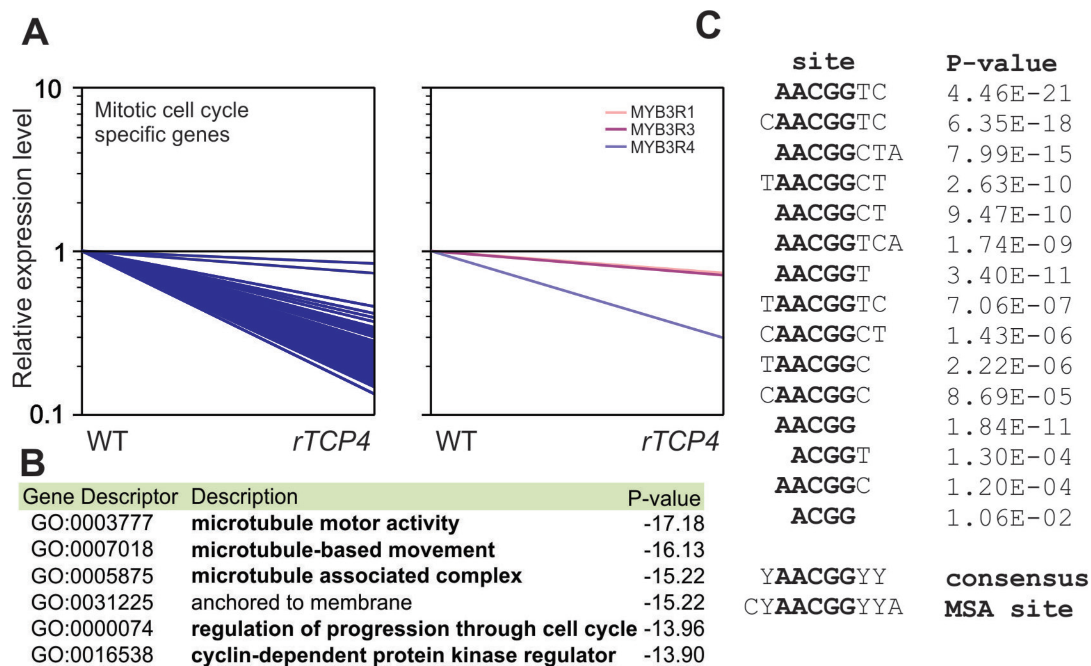

Figure 2 Repression of Cell Cycle Genes in *rTCP4* Plants.

(A) Comparison of expression levels of mitosis-specific genes in ATH1 arrays of wild-type and *rTCP4* apices. Right: expression of three R1R2R3 MYBs in *rTCP4* microarrays.

(B) Results of GO term analysis of down-regulated genes in *rTCP4* apices. Categories highlighted in bold are related to cell division and cell cycle.

(C) Analysis of over-represented sequences in promoters of genes down-regulated in *rTCP4*.

(E) Disassembled rosettes of *soj8* and WT. Leaves of *soj8* are smaller and more lanceolated than WT. The angle between the leaf blade and the petiole (indicated by yellow line) was measured in the fourth leaf.

(F) Measurement of leaf area of fully expanded first leaves in WT Columbia, and heterozygous and homozygous *soj8* lines.

(G) Measurement of palisade parenchyma cell size of leaf No. 1 in WT, heterozygous, and homozygous *soj8* lines.

(H) Cell numbers of leaf No. 1 determined for WT, heterozygous, and homozygous *soj8* lines.

(I) First leaves of Columbia WT, *soj8* heterozygous, and *soj8* homozygous plants as seen after fixing in FAA and clearing in chloral hydrate.

analyzed the array data for over-represented motives in the promoters of the genes down-regulated in the *rTCP4* samples using the element software package (Mockler et al., 2007). We found that the most enriched boxes clustered together generating the consensus sequence YAACGGYY (Figure 2C), which resembles the MSA (Mitotic Specific Activator) sequence, present in genes active during mitosis (Menges et al., 2005; Haga et al., 2007).

MSA sites are recognized by R1R2R3-Myb proteins with the *Arabidopsis* genome containing five related genes (Haga et al., 2007). Three R1R2R3-Mybs, present in the ATH1 microarrays, showed decreased transcript levels in the *rTCP4* samples (Figure 2A). R1R2R3-Mybs redundantly regulate G2-M phase genes and cytokinesis (Haga et al., 2007, 2011). Mutations in two of them (*MYB3R1* and *MYB3R4*), which are also reduced by *rTCP4*, have been found to cause a reduction in plant size and stature (Haga et al., 2011).

It has been shown that miR319-regulated TCPs stimulate cell differentiation (Efroni et al., 2008; Sarvepalli and Nath, 2011). Our microarray analysis performed on dissected plant apices, however, shows that TCP4 has a strong effect on cell proliferation genes. Then, it might be possible that the repression of cell proliferation is an early event in the function of the miR319-regulated TCPs, which might contribute to the accelerated differentiation of *rTCP4* plants. The TCP4-binding site (Schommer et al., 2008; Aggarwal et al., 2010) is different to the MSA element, and we did not detect it in the promoters of the R1R2R3 MYBs, so we considered the repression of the mitosis-specific genes to be an indirect outcome of TCP action probably through the activation of genes that act as repressors of cell proliferation.

Potential Links between TCP4 and Pathways Repressing Leaf Growth

Previous results have shown that TCP4 can up-regulate *LOX2* and JA levels (Schommer et al., 2008; Gonzalez et al., 2010), as well as micro RNA miR396 (Rodriguez et al., 2010). In principle, both high JA and miR396 can repress cell proliferation (e.g. Pauwels et al., 2008; Zhang and Turner, 2008; Rodriguez et al., 2010), but their actual participation in the miR319/*TCP4* pathway has not been evaluated so far.

Therefore, we first crossed *soj8* with *aos*, a mutant in the JA biosynthetic pathway (Park et al., 2002). We found that the plants homozygous for both mutations were similar to *soj8* (Figure 3A), indicating the *soj8* allele can still repress leaf growth in a mutant background lacking MeJA (Figure 3B). We also treated *soj8* exogenously with MeJA and again found similar growth inhibition effects in both mutants and wild-type plants (Figure 3C). These results suggest that JA signaling is not affected in *soj8* plants and that JA probably does not contribute significantly to the

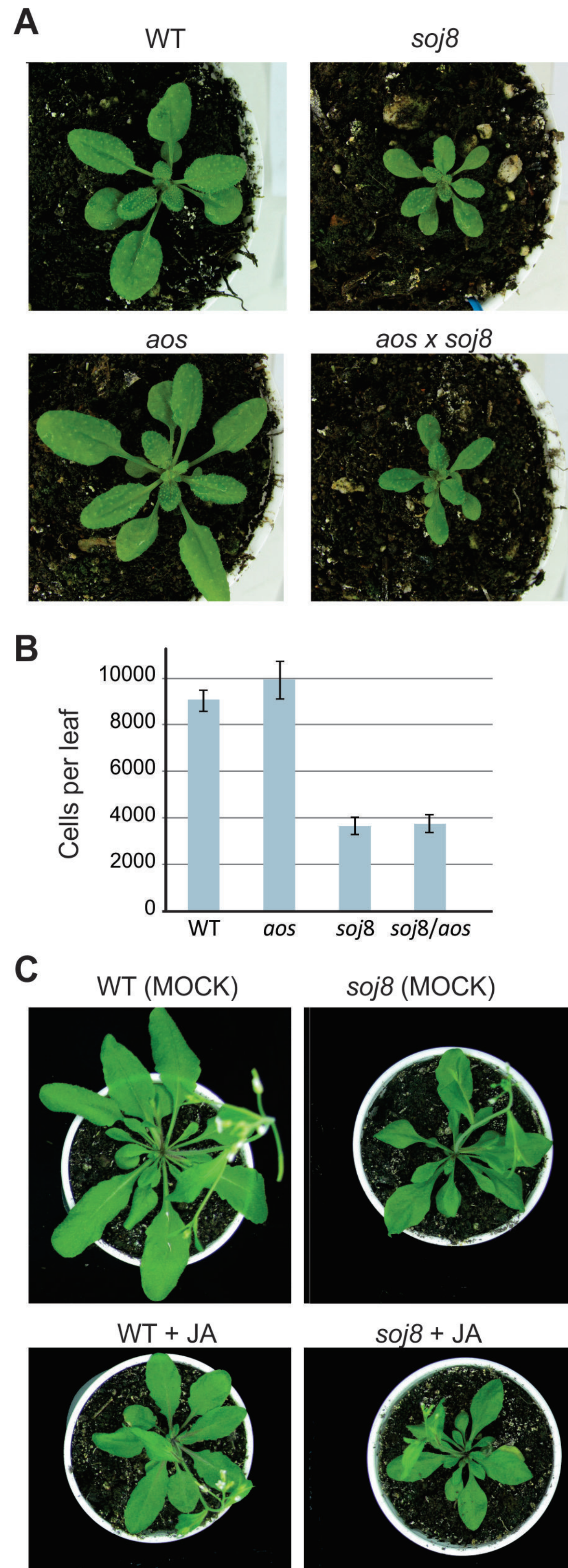

Figure 3 Effect of MeJA on Cell Division in *soj8* Plants.
(A) Four-week-old rosettes of wild-type (WT), *soj8*, *aos*, and *aos*/*soj8* plants as indicated in the figure.

reduction of leaf growth caused by a moderate increase in *TCP4* levels even though it has been shown to be a repressor of cell division. It is plausible that the slight increase in *TCP4* levels observed in *soj8* caused an increase in JA that is not sufficient to affect leaf size, but we cannot rule out the participation of JA in the stronger developmental defects observed in plants with higher *TCP4* levels.

TCP4 Partially Represses Cell Proliferation through miRNA miR396

Then, we evaluated the genetic interaction between *soj8* and the miR396-Growth-Regulating Factor (GRF) system. MiR396 regulates *GRF* transcription factors and its overexpression decreases the number of cells in leaves (Liu et al., 2009; Rodriguez et al., 2010; Wang et al., 2011; Mecchia et al., 2012). Although it has been previously shown that high *TCP4* activity increases miR396 levels (Rodriguez et al., 2010), the contribution of miR396 to the developmental defects caused by *rTCP4* has not been evaluated so far.

To do this, we first characterized transgenic plants carrying different versions of *GRF3*, a known target of miR396 (Jones-Rhoades and Bartel, 2004). We analyzed a wild-type transgene and a miR396-insensitive transgene (*rGRF3*) with synonymous mutations in the miRNA-binding site as described before (Rodriguez et al., 2010). Plants carrying additional copies of the wild-type or miR396-resistant *GRF3* had bigger leaves than control plants (Figure 4A and 4B), in agreement with the role of the GRFs as promoters of leaf growth (Kim et al., 2003; Horiguchi et al., 2005; Rodriguez et al., 2010; Debernardi et al., 2012, 2014).

We also observed that *rGRF3* was more efficient in the promotion of growth than its wild-type counterpart (not shown), as expected as *rGRF3* is not repressed by miR396, similarly to the previous results with *GRF2* (Rodriguez et al., 2010). Here, we selected transgenic lines carrying *GRF3* and *rGRF3* transgenes that caused a similar increase in leaf size and crossed them to *soj8* (Figure 4A and 4B). We observed that *soj8* x *rGRF3* leaves were significantly larger than *soj8* x *GRF3*, indicating that part of the reduction of leaf size caused by *soj8* is due to the induction of miR396 (Figure 4A and 4B). The recovery of the size of the organ by *rGRF3* is in agreement with the induction of miR396 and the repression of *GRF3* observed in *soj8* (Figure 4C). This increase in miR396 observed in *soj8* plants is able to inactivate the wild-type *GRF3* allele, but not the miR396-insensitive version.

Interestingly, the shape of the leaves of *soj8* x *rGRF3* was still reminiscent of *soj8*, with small angles between the petiole and the leaf lamina (Figures 1 and 4B). These results

(B) Number of cells in leaf one of the different genotypes.
(C) Growth inhibitory effects of exogenous MeJA treatment on WT and *soj8* plants.

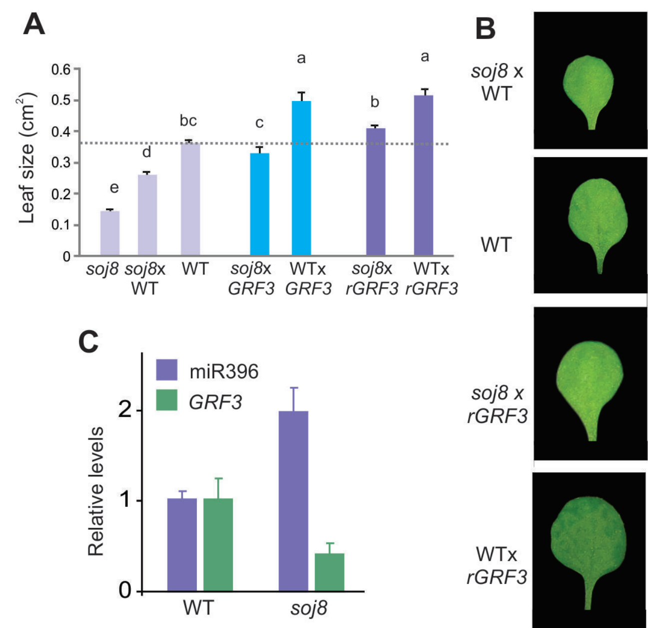

Figure 4 Regulation of Leaf Size by TCP4 through miR396/GRF Module.

(A) Size of first leaf in crosses of *GRF3*- and *rGRF3*-expressing plants to *soj8* plants, and the respective controls. Small letters over bars in (A) identify statistically significant different groups.

(B) Pictures of the first leaves of representative crosses.

(C) Measurement of miR396 and *GRF3* by RT–qPCR wild-type and *soj8* plants.

indicate that TCP4 induces changes in both leaf shape and size, and that *rGRF3* might in principle rescue the leaf size pathway only.

MIR396b Has a Functional TCP4-Binding Box in Its Promoter

Next, we explored the link between TCP4 and miR396 in developing leaves. TCP4 binds to the DNA sequence GGACCA(C), or its reverse complement sequence (G) TGGTCC (Schommer et al., 2008). There are two genes encoding miR396 in *A. thaliana*: *MIR396a* and *MIR396b*. Inspection of the sequences upstream of the foldback precursors revealed that *MIR396b* has a GTGGTCC box 120 nt upstream of the miRNA precursor. In contrast, we did not find this sequence in the *MIR396a* genomic context.

Analysis of *MIR396a* and *MIR396b* reporters in seedlings indicated that *MIR396a* was mainly expressed in the vascular system of the areal part of the plant and roots (Figure 5A), while *MIR396b* was expressed strongly in leaves (Figure 5B), with a pattern following the proliferation arrest front (Debernardi et al., 2012). Mature miR396a and miR396b differ in their last nucleotide, and deep sequencing analysis of small RNA libraries indicated that they are

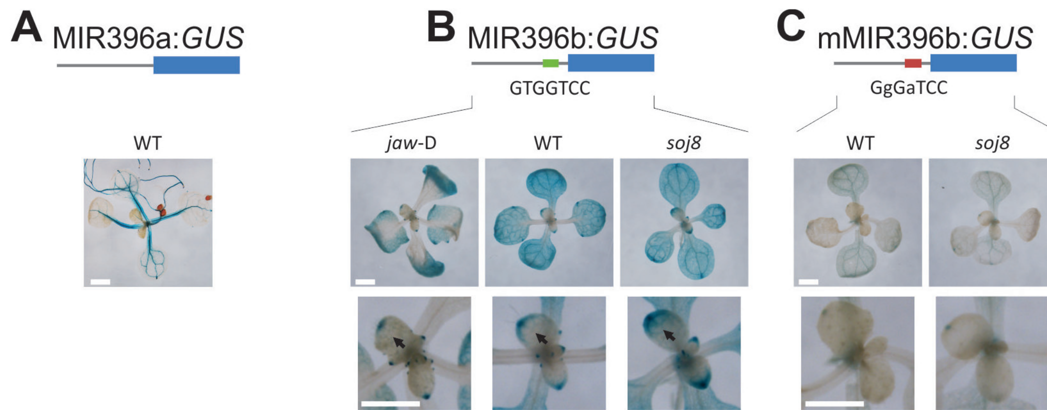

Figure 5 Expression of *MIR396* Reporters.

(A–C) GUS staining of 10-day-old seedlings. (A) *MIR396a* wild-type promoter fragment fused to GUS. (B) *MIR396b* wild-type promoter fragment, TCP-binding site indicated, sequence given in capital letters. (C) *MIR396b* promoter with mutations in TCP4-binding site, mutated bases indicated by small letters. GUS staining was carried out in the genetic background indicated above each photo which was wild-type (WT), *jaw*-D, or *soj8.* The arrows highlight the expression of MIR396b:*GUS* in a developing leaf. Scale bars = 1 mm.

expressed in different tissues, with miR396b being more expressed in the areal parts of the plant (shoots and flowers), while miR396a was more expressed in roots (Jeong et al., 2013). Therefore, the analysis of *MIR396* reporters is in agreement with the small RNA data indicating that *MIR396b* is probably the most highly expressed miR396 family member in *Arabidopsis* leaves.

Then, we analyzed the activity of the *MIR396b* reporter in the context of the *jaw*-D mutant, which has strongly reduced TCP activity (Palatnik et al., 2003). We observed a clear down-regulation of the *MIR396b*-reporter activity in this mutant (Figure 5B). In addition we also crossed the reporter to *soj8* plants that have a slightly increased TCP4 activity. In this case, we observed an increase of the area expressing MIR396b activity in developing leaves (Figure 5B).

Next, we decided to compare the expression of a wild-type and a mutant *MIR396b* promoter–GUS reporter, in which two mutations were introduced in the TCP4-binding site (*mMIR396b*, mutated TCP4-binding site (G) gGaTCC) (Figure 5B and 5C). Nine out of 14 T1 seedlings with the wild-type promoter construct showed GUS activity in expanding and mature leaves (Figure 5B). In contrast, plants harboring the mutated TCP-binding box showed a reduced GUS activity restricted mostly to cotyledon veins and roots (eight out of 14 primary transgenic plants) (Figure 5C) or no staining at all (six out of 14 primary transgenic plants), showing the importance of this site in the transcriptional regulation of *MIR396b*, and suggesting a direct regulation of miR396 expression by TCP4. We also analyzed the activity of the mutated *MIR396b* reporter in the background of the *soj8* and saw that the expression pattern did not change (Figure 5C), as would be expected for elimination of the TCP-binding site.

Direct Activation of *ICK1*/*KRP1* by TCP4

The previous data showed that the activation of miR396 was partially responsible for the repression of cell proliferation caused by TCP4. To characterize this process in more detail, we performed ATH1 arrays of *35S:MIR396b*-overexpressing plants (Figure 6A). Interestingly, the shape of *35S:MIR396b* rosette leaves is slightly lanceolated as in *soj8* plants (Figures 1A, 1E, and 6A). Previous arrays of *rTCP4* were performed on RNA derived from vegetative apices of plants grown in short days, so we carried out ATH1 microarrays of *35S:MIR396b* samples collected in a similar way. However, whereas for the arrays of *35S:MIR396b* one stable line was chosen for the sample collection, in the case of the *rTCP4* arrays a mixture of primary transgenic plants was the source for the array, many of them with stronger phenotypes (Figure 1B) (Schommer et al., 2008).

Differentially expressed genes compared to wild-type were selected by both statistical and common variance as performed before (Supplemental Tables 1–4). Comparing the data sets, we observed that *rTCP4* expression modified transcription of more genes than *35S:MIR396b* expression (Figure 6B and 6C). That the *rTCP4* arrays were performed on different primary transgenic plants, some of them with stronger phenotypes than *35S:MIR396b*, might explain part of this effect. On the other hand, TCP4 is an upstream regulator of *MIR396b* and has been implicated in several different pathways that are probably independent of miR396 activity and that go beyond cell proliferation, which can explain the larger number of genes affected in *rTCP4*.

Interestingly, we found that approximately half of the genes that were induced by miR396 were also induced by *rTCP4* (Figure 6C, and Supplemental Tables 2 and 4). In the case of repressed genes, approximately 66% of the

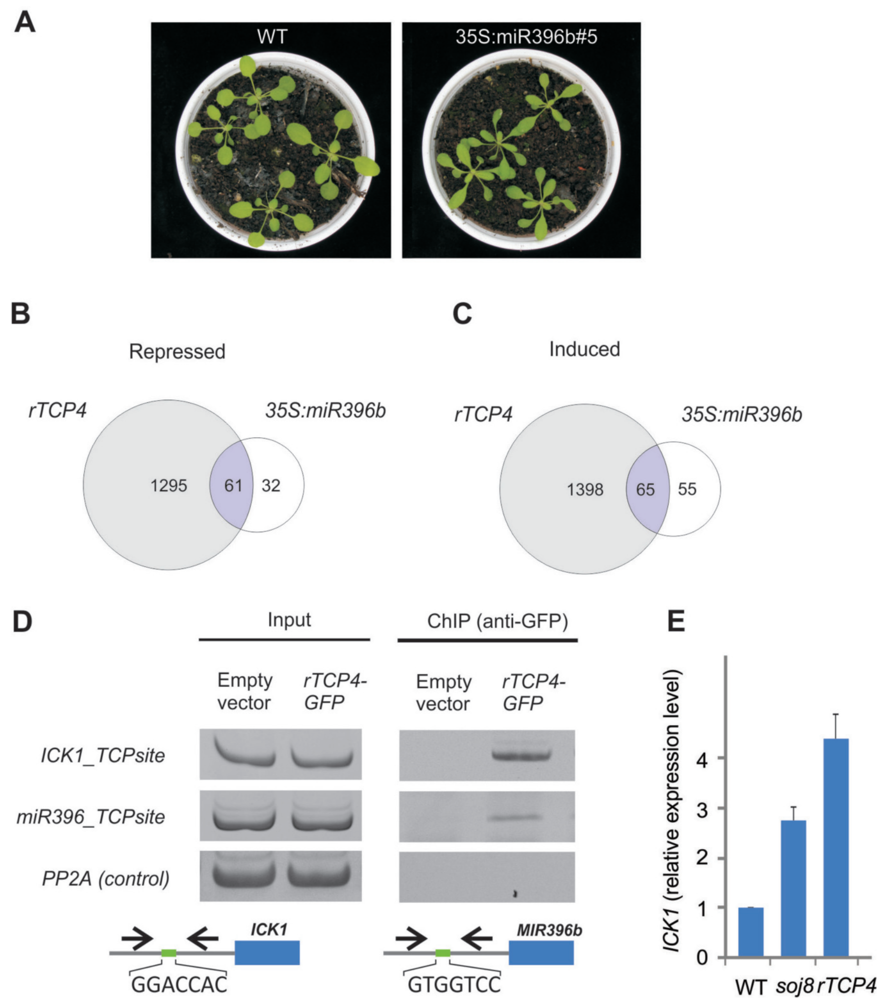

Figure 6 Potential Links between TCP4 and Cell Proliferation.

(A) Wild-type (WT) and *35S:MIR396b* plants grown in short days as used for harvesting material for array experiments.

(B) Genes down-regulated in *rTCP4* and *35S:miR396b* arrays.

(C) Genes up-regulated in *rTCP4* and *35S:MIR396b* arrays; group of overlapping genes indicated in lilac.

(D) Gel after PCR of Chromatin Immunoprecipitation experiment with *rTCP4–GFP* plants showing direct binding of TCP4 to the promoters of *ICK1/KRP1* and *MIR396b*. Binding sites in *ICK1/KRP1* and *MIR396b* promoters are indicated in the graph under the gel.

(E) Relative expression levels of *ICK1/KRP1* transcript in WT, *soj8*, and *rTCP4* plants.

genes that were down-regulated in *35S:MIR396b* were also down-regulated in *rTCP4* apices compared to wild-type tissue (Figure 6B, and Supplemental Tables 1 and 3). These results are in agreement with our previous observations indicating that part of the role of *rTCP4* in the repression of cell proliferation is performed through the activation of miR396. Not all genes that are induced or repressed in *35S:MIR396b* plants are changing their expression pattern in *rTCP4* plants. This may be due to effects of overexpressing miR396 from the strong 35S promoter or that 35S is ubiquitously activating miR396, also outside of the endogenous expression domain of the microRNA.

The previous results also showed that rTCP4 can repress cell proliferation independently of miR396. In our experimental system, *LOX2* was induced by rTCP4 but not by high levels of miR396, indicating that TCP4 can take influence on different unconnected pathways for regulating cell division. Therefore, to look for other TCP4-specific pathways involved in the repression of cell proliferation, we analyzed also genes up-regulated by the transcription factor that did not change in the *35S:MIR396b* arrays. We additionally required that the genes induced by *rTCP4* harbor a TCP4-binding site, GTGGTCC, or its reverse complemented GGACCAC, in their promoters (Supplemental Table 5).

One gene that fulfilled these criteria was *ICK1/KRP1*, as it was induced three- to four-fold in *rTCP4* and also *soj8* plants (Figure 6E). Like *rTCP4* and *soj8*, plants with higher

levels of *ICK1* have leaves smaller than wild-type (Wang et al., 2000; Malinowski et al., 2011).

To validate the binding of the TCP4 protein to the *ICK1/KRP1* promoter, we performed ChIP–PCR experiments of plants carrying a *TCP4:rTCP4–GFP* transgene using vegetative apices as a sample. We found that TCP4 indeed binds to the predicted region of *ICK1/KRP1* (Figure 6D). We also tested the binding of TCP4 to the *MIR396b* promoter, further confirming the direct regulation of *MIR396b* by TCP4 (Figure 6D). Altogether, the results show that miR319-regulated TCPs can directly activate different pathways that inhibit cell division and the effects observed in leaf size by modifying the TCP levels are likely the result of the concert action of more than one of these pathways (Figure 7).

Conclusions

Here, we have shown that TCP4 has a primary function repressing cell proliferation—an activity that probably depends on the activation of different repressors, one of them being *MIR396b* (Figure 7). MiR396 represses *GRFs*, which are positive regulators of leaf growth (Kim et al., 2003; Horiguchi et al., 2005; Rodriguez et al., 2010; Wang et al., 2011; Mecchia et al., 2013; Debernardi et al., 2014). We think that the repressors of cell proliferation activated by TCP4 will ultimately repress R1R2R3-Mybs and mitosis-specific genes. Interestingly, the strong phenotypes caused by rTCP4 involved cotyledon fusions and embryo patterning defects (Palatnik et al., 2003)—the same defects as observed in *myb3r1* and *myb3r4* double mutants (Haga et al., 2011). However, it is important to mention that intermediate steps in the repression of cell proliferation by TCP4 remain to be elucidated, as, for example, the gene networks directly regulated by the miR396-regulated GRFs are currently unknown.

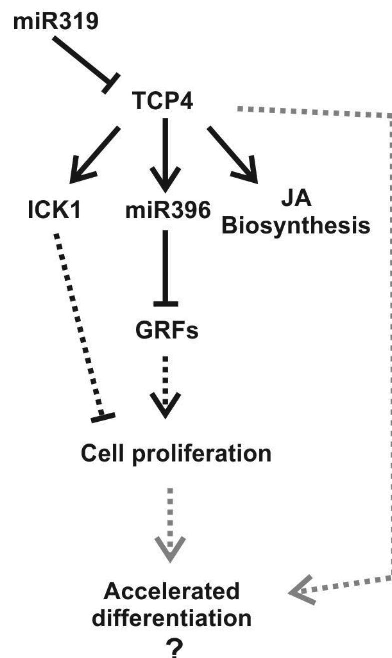

Figure 7 Scheme of the Role of TCP4 in the Regulation of Cell Proliferation.

MiR396 has a specific pattern of expression during leaf development, accumulating in the distal part of the organ and with the age of the leaf (Rodriguez et al., 2010; Debernardi et al., 2012). This pattern is disrupted after mutating the TCP4-binding box in the *MIR396b* promoter, indicating that the TCPs are important regulators of miR396 during *Arabidopsis* development. It has also been shown that miR396 represses cell proliferation in response to UV light (Casadevall et al., 2013). It will be interesting to test whether TCP4 participates in this activation. Two other miRNAs, miR156 and miR172, sequentially regulate developmental timing (Wang et al., 2009; Wu et al., 2009). It is conceivable that a cascade of two other miRNAs regulating transcription factors, miR319 and miR396, is involved in the control of cell proliferation during the development of each leaf.

It has been previously shown that miR319-regulated TCPs induce *LOX2* expression and JA biosynthesis (Schommer et al., 2008), which is a known inhibitor of the cell cycle (Pauwels et al., 2008; Zhang and Turner, 2008). In our experimental set-up, we did not find an obvious contribution of this pathway to the control of cell proliferation. However, we have used *soj8* mutants, which have less than a two-fold activation of *TCP4* levels and, therefore, we cannot discard the participation of JA in the inhibition of the cell cycle in plants with higher *TCP4* levels.

In addition, we have found that TCP4 directly activates *ICK1/KRP1*, a core gene involved in the progression of the cell cycle (reviewed in Inze and De Veylder, 2006; Gutierrez, 2009). High levels of *ICK1/KRP1* inhibit cell proliferation and reduce leaf growth (Wang et al., 2000). Interestingly, *ICK1/KRP1* was not induced by miR396 overexpression, indicating that TCP4 can inhibit cell proliferation by different mechanisms. This is in agreement with the miR319-regulated TCPs being upstream of miR396 and regulating other pathways as well. It has also been shown that miR319-regulated TCP3 controls the miR164-CUC network (Koyama et al., 2007), which has been involved in the control of organ separation, leaf margin formation, and cell proliferation (Laufs et al., 2004; Nikovics et al., 2006; Hasson et al., 2010; Kawamura et al., 2010; Hasson et al., 2011).

Detailed analysis of knockdowns in the miR319-regulated *TCP*s, including *TCP4*, has shown that they have a delay in cell differentiation, while higher levels or activated versions accelerate the process (Efroni et al., 2008; Sarvepalli and Nath, 2011). It might be plausible that the repression of cell proliferation that we observed here triggers part of the differentiation programs of the cells, so that the two processes are at least partially linked (Figure 7).

METHODS

Plant Material

Plants were grown at 22°C under long days (16 h light/8 h dark) except for the plants used in the microarray experiments, that were grown under short days (8 h light/16 h dark). Wild-type was Columbia-0. The *soj8* mutant was described earlier (Palatnik et al., 2007).

Microarray Analyses

Total RNA was extracted from dissected vegetative apices of plants grown in SD condition for 20 d using the RNeasy plant mini kit (QIAGEN). Microarray analyses using the Affymetrix ATH1 platform were performed on two biological replicates (NCBI GEO GSE53839). Expression data were processed with Robin software (Lohse et al., 2010) with the following setting—analysis strategy: Limma; normalization method: gcrma; *P*-value cut-off value for significant differential expression: 0.05. For the collection of apices, plants were dissected under a stereomicroscope and all leaves with visible petioles were removed from the sample. Very young leaf primordia were therefore included in the apical region and collected directly into collection tubes floating in liquid N_2.

Expression Analyses

RNA was extracted using TRIzol (Invitrogen). 1 μg of total RNA was treated with RQ1 RNase-free DNase (Promega). Next, first-strand cDNA synthesis was carried out using the SuperScript™ Reverse Transcriptase (Invitrogen) using oligo dT(15)V. PCR reactions were carried out in a Stratagene Mx3000P QPCR system using SYBR-Green I (Roche) to monitor cDNA synthesis. Relative transcript levels were determined in each sample, using *PROTEIN PHOSPHATASE 2A* (At1g13320) to normalize to. β-glucoronidase assays were carried out as described earlier (Donnelly et al., 1999).

Measurements of Leaf Size

Leaves subjected to measurement of area were detached from the rosette and photographed in the presence of a ruler. The images were analyzed using ImageJ software.

Measurements of Cell Size and Number

First and fifth fully expanded leaves were photographed and then fixed in FAA for 24 h and cleared in chloral hydrate. Leaves were mounted on microscopy slides and pictures of the cleared palisade mesophyll cells were taken with a microscope. Ten palisade cells each were measured in different areas of the leaves using ImageJ software (http://rsb.info.nih.gov/ij/).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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