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THE ROLE OF THE MicroRNA156/*SPL* PATHWAY DURING THE PRIMARY ROOT GROWTH OF *Arabidopsis thaliana*

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Palavras-chave: *Arabidopsis thaliana*; Fitohormônios; Raíz primaria; Sistema radicular; miR156.

CONTENTS

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ABBREVIATIONS

ARF	:	Auxin Response Factors
Col-0	:	*Arabidopsis* wild-type plants in Columbia ecotype
dpg	:	Days-post-germination
LR	:	Lateral root
MIM156	:	Transgenic plants with highly reduced levels of the available mature miR156
miR156/*SPL*	:	Genetic pathway controlled by the microRNA156 and its targets, members of the *SPL* family
miRNAs	:	MicroRNAs
p35S::MIR165A	:	Transgenic plants overexpressing the *MIR156A* gene
PR	:	Primary root
RMS	:	Root meristem size
SPL	:	*SQUAMOSA Promoter-Binding Protein-Like*
WT	:	Wild-type plants

LIST OF FIGURES

LIST OF APPENDICES

ABSTRACT

Root system (RS) is important for anchorage and for up-taking water and nutrients. In eudicots, such as *Arabidopsis*, the primary root (PR) growth is affected by phytohormones, especially auxin controlling cell division and cytokinin mediating cell differentiation; also, microRNAs (miRNAs), a subset of small RNAs that post-transcriptionally regulate their targets, regulate the PR growth. The microRNA156 (miR156) and its targets, members of the *SQUAMOSA Promoter-Binding Protein-Like* (*SPL*) family, constitute a genetic pathway that regulates several developmental processes including root development; however, during PR growth it was not observed the effect of the miR156/*SPL* pathway, and the interplay with auxin/cytokinin; therefore, we evaluated this interaction during the root meristem size (RMS)-mediated PR growth in *Arabidopsis*. Using molecular and genetic tools, we analyzed the *MIR156* and *SPL* gene expressions, the PR length, the RMS, the cell division rates, and the auxin/cytokinin responses during PR growth. *MIR156* and *SPLs* genes have opposite expression patterns. High levels of mature miR156 (in *p35S::MIR156A* seedlings) lead to shorter PR, reduced RMS, lower rates of cell division, lower and higher auxin and cytokinin responses, respectively; conversely, reduce levels of the available mature miR156 (in *MIM156* seedlings)

lead to opposite effects. De-regulation of the *SPL10* (in miR156-resistant version of *SPL10*) promotes longer PR, larger RMS, higher CYCLIN G2-M-specific *CYCLINB1;1* (*CYCB1;1*) expression, and reduce cytokinin responses evaluated by *ARR1* expression, *nTCS:GFP* and *pARR5:GUS* reporters, than Col-0. Our data suggest that *SPL10* de-regulation increases the cell division-mediated RMS and consequently promotes the PR growth by altering cytokinin responses in *Arabidopsis.*

KEY WORDS: *Arabidopsis*, Root system, Primary root, phytohormones, MiRNAS, miR156, *SPL.*

RESUMO

O sistema radicular (SR) é importante pela ancoragem e obtenção de água e nutrientes. Em eudicotiledôneas, como *Arabidopsis*, o crescimento da raiz primária (RP) é afetado por fitormônios, especialmente pelo balanço entre auxina que controla a divisão celular, e citocinina que modula a diferenciação celular; também, os microRNAs (miRNAs), um sub-conjunto de pequenos RNAs que regulam pós-transcricionalmente seus alvos, regulam o crescimento da RP. O microRNA156 (miR156) e seus alvos, membros da família *SQUAMOSA Promoter-Binding Protein-Like* (*SPL*), constituem uma via genética que regula vários processos do desenvolvimento, incluindo desenvolvimento da raíz; porém, durante o crescimento da RP, não foi observado o efeito da via miR156/*SPL*, e da interação com auxina e citocinina; assim, foi avaliada essa interação durante o crescimento da PR regulado pelo tamanho do meristema da raiz (TMR) em *Arabidopsis.* Usando ferramentas genéticas e moleculares foi analizada a expressão de genes *MIR156* e *SPL*s, o comprimento da RP, o TMR, as taxas de divisão celular, e as respostas de auxina e citocinina durante o crescimento da RP. Os genes *MIR156* e *SPL*s possuem padrões de expressão opostos. Níveis altos do miR156 (nas plântulas *p35S :: MIR156A*), leva a menor comprimento da RP, TMR reduzido, menores taxas de divisão celular, respostas mais baixas e

altas à auxina e citocinina respectivamente; em contraste, níveis severamente reduzidos do miR156 maduro disponível (nas plantas *MIM156*) conducem a efeitos opostos. Des-regulação da *SPL10* (em plantas com a versão resistente ao miR156, *rSPL10*) promove crecimento da RP, maior TMR, maior expressão do gene *CYCLINB1,* redução da resposta à citocinina, avaliada pela expressão de *ARR1,* e dos genes repórteres *nTCS::GFP* e *pARR5::GUS,*do que Col-0. Os nossos dados sugerem que a des-regulação da *SPL10* incrementa o TMR pelo aumento nas taxas de divisão celular e, consequentemente, aumentando o comprimento da RP, pela redução das respostas à citocinina em *Arabidopsis.*

Palavras chave: *Arabidopsis*, Sistema radicular, Raíz primaria, Fitohormônios, MiRNAS, miR156, *SPL*.

1. INTRODUCTION

The root is the organ of the plant's body that commonly lies below the surface of the soil. Its functions include, mainly, supply plants with micro and macronutrients, water, anchorage, and phytohormone biosynthesis; in addition, root functions may include energy storage organ and clonal propagation. Thus, the root has an important role in yield and overall plant productivity (Osmont *et al*., 2007; Lynch, 1995). In the model plant *Arabidopsis thaliana*, as in eudicots, the root system is composed by a primary root (PR), lateral roots (LR), and eventually adventitious roots (Figure 1A; Boyes *et al*., 2001). Along the longitudinal axis, the PR displays three developmental zones into a simple structure composed of the stele surrounded by four one-cell layers (Figure 1B-D), and its growth is sustained by the activity of the root meristem, a sustainable and self-renewable system which activity depends on different factors, including phytohormones-controlled biochemical routes, and microRNAs-regulated genetic pathways (Xue *et al*., 2017; Dello Ioio *et al*., 2008).

The phytohormones-controlled biochemical routes are essential for PR growth by controlling the root meristem size (RMS). The RMS is a critical factor to ensure the suitable PR growth, and it is specially affected by the antagonistic

effects of auxin and cytokinin (Dello Ioio *et al*., 2008). Auxin is important for plant patterning; in roots, it establishes positional information for cell fate decisions and maintains the root meristem activity (Blilou *et al*., 2005). The meristem activity is characterized by cell divisions, and it depends on cell cycle progression. The cell cycle has two important transition steps, the G1-S and the G2-M, which are modulated by members of the *Cyclin-Dependent Serine-Threonine* protein kinase family, a family of proteins associated with cell cycle (Tank and Thaker, 2011). Among them, the CYCLIN G2-M-specific *CYCLINB1;1* (*CYCB1;1*) expression was clearly identified into the root meristematic zone and directly associated with the RMS (Ferreira *et al*., 1994).

The cell divisions on meristematic zone are modulated by an auxin gradient; while high levels of auxin are found in the proximal meristem low levels are found on the distal meristem (Petersson *et al*., 2009; Jurado *et al*., 2010). These auxin gradients are partially generated by the PIN-FORMED (PIN) auxin-efflux carriers, which funnel auxin efflux across cells (Vieten *et al*, 2005; Wiśniewska *et al*, 2006); among them, the PIN1, PIN3, and PIN7 proteins have been shown to be essential for controlling the RMS (Figure 1B; Dello loio *et al*., 2008).

Besides auxin that promotes cell division, cytokinin mediates cell differentiation by antagonizing auxin on transition zone and, consequently,

contributes to establish the RMS. The cytokinin-controlled RMS involved the activation of the nucleus-localized type-B *ARABIDOPSIS RESPONSE REGULATORS* (ARRs) ARR1 and the ARR12 transcription factors with *ARR1* being a critical factor to determine the root meristem size. Both, *ARR1* and *ARR12* are expressed at transition zone, where they repress the expression of the *PIN* genes through SHY2/IAA3 (SHY2) protein; a negative regulator of PINs transporters by forming heterodimers with the auxin response factor (ARF) family of transcription factors, preventing the activation of auxin-responsive genes (Tian *et al.,* 2002). This model proposes that cytokinin and auxin antagonistically interact at the transition zone to balance cell differentiation with cell division (Figure 1B), which is essential to stabilize the RMS and to ensure continuous and proper root growth (Dello loio *et al*., 2007; 2008).

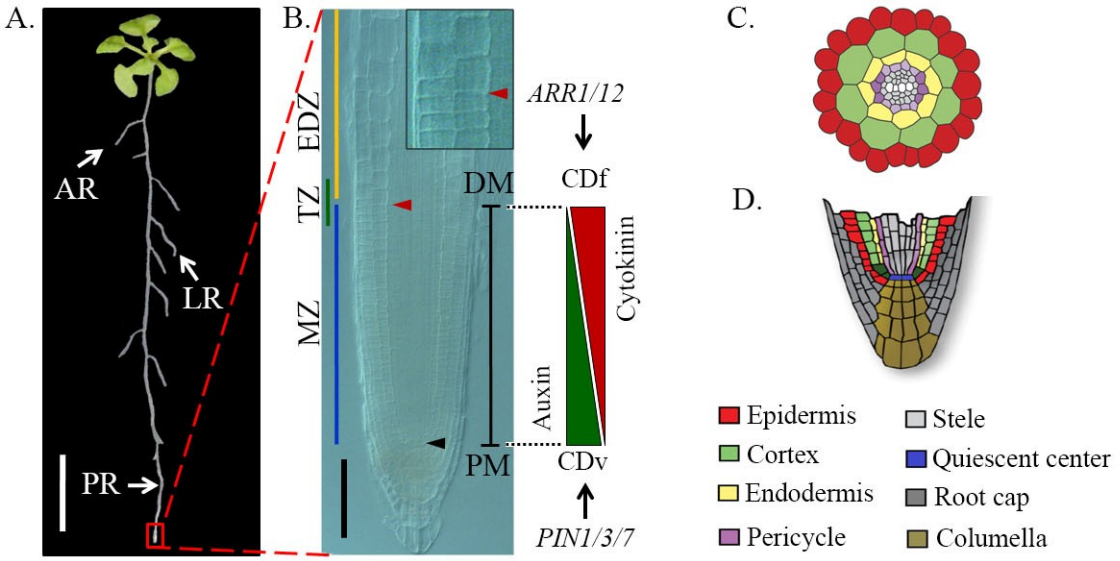

Figure 1. The root system in *Arabidopsis thaliana.* A.: representative picture of the root system in Col-0 at 12-days-post-germination (dpg), indicating the adventitious root (AR), lateral root (LR), and the primary root (PR); scale bar: 1 cm. B.: Light microscope picture of the longitudinal view of root meristem from Col-0 at 10-dpg, showing the elongation-differentiation zone (EDZ), transition zone (TZ) and the meristematic zone (MZ); red arrowhead indicates the TZ (highlighted in the upper small box), and black arrowhead indicates the quiescent center; on MZ is also indicated the proximal meristem (PM) and the distal meristem (DM), scale bar: 100 µm; the green-red scheme illustrates the auxin-cytokinin gradients on MZ, being auxin promoting division (CDv) mainly through PIN1/3/7 proteins, and cytokinin mediating cell differentiation (CDf) by *ARR1/12* genes. C-D: Cross-sectional and longitudinal illustration of the EDZ and the root tip respectively, showing the different one-cell layers.

Other essential factors that contribute for the RMS and, consequently, a suitable PR growth are the genetic pathways controlled by microRNAs (Xue *et al.*, 2017; Rodriguez *et al.*, 2015). MicroRNAs (miRNAs) are an endogenous subset of hairpin-derived small RNA with 21-24 nucleotides long. They negatively regulate the gene expression of their targets by RNA cleavage, translational inhibition, or chromatin modifications, comprising one of the most abundant classes of gene regulatory molecules in multicellular organisms (Axtell, 2013). In plants, this tiny regulatory RNAs are derived from the processing of

helical regions of RNA precursors; this process was previously described (Figure 2A) but, in summary, the miRNA mature molecule is derived from one arm of fold-back precursor that comes from the RNA polymerase II-dependent transcription of *MIR* genes, which binds to the RNA-induced silencing complex (RISC), and will regulate the gene expression of its complementary RNA target mostly by the ARGONAUTE (AGO)-directed cleavage or the translational inhibition (Bartel, 2004; Kurihara and Watanabe, 2004).

In plants, miRNAs are involved in different genetic pathways affecting many different processes as phase change, development of leaves and reproductive organs (Huijser and Schmid, 2011; Xie *et al*, 2012). In roots, especially in *Arabidopsis*, several miRNAs-controlled genetic pathways were reported modulating the root development (Figure 2 B). For instance, during the LR development it was observed that, while the miR160 and miR390 promote the LR production and elongation respectively, the miR164 and miR167 negatively regulate the LR development. The miR160 regulates the *AUXIN RESPONSE FACTOR10* (*ARF10*)*, ARF16,* and *ARF17* transcription factors (Rhoades *et al*., 2002). Among them, *ARF17* seems to be involved in root development because disrupting in *ARF17* mRNA levels leads to abnormalities in LR production (Mallory *et al*., 2005). The miR390 also regulates members of the *ARF* family through the trans-acting small-interference RNAs (tasiRNA). The miR390 cleaves the non-coding *TAS3* precursor; the cleaved product is polymerized into RNA/RNA double-strand, and then cleavage by DICER-Like4

(DCL4), generating the tasiRNAs. This small interference RNAs will drive the cleavage of the *ARF2*, *ARF3*, and *ARF4* transcripts through ARGONAUTE 7 (AGO7). In transgenic plants that over express the *TAS3* precursor (35S:TAS3a) the length of LRs was longer while in the *tas3a-1* mutant was shorter than wild-type controls, suggesting that the miR390 positively regulates the LR elongation (Marin *et al*., 2010).

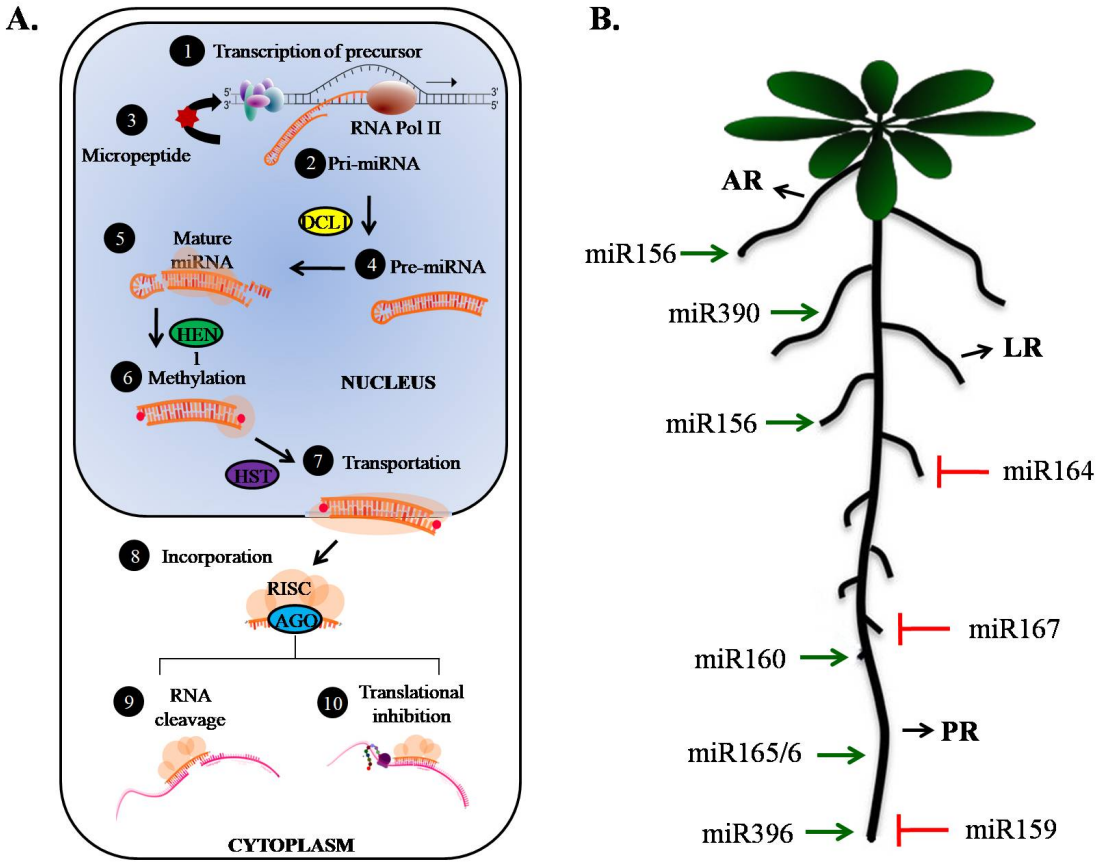

Figure 2. Biogenesis and miRNAs in *Arabidopsis* root growth and development. A.: the biogenesis of the miRNAs in plants. After the RNA polymerase II-mediated transcription of precursor (1), it is generated a single stranded precursor named pri-miRNA (2). In some cases, this precursor encodes a micro-peptide that enhance their own transcription (3; Lauressergues *et al*., 2015); but in the canonical biogenesis, the pri-miRNA folds itself into a hairpin structure; this structure is cleaved in two-steps by the DICER-LIKE 1 (DCL1)

enzyme, producing a pre-miRNA (4) and then a duplex miRNA/miRNA (5). The duplex suffers a HUA ENHANCER 1 (HEN1)-mediated methylation (6), and a HASTY (HST)-mediated transportation from nucleus to cytoplasm (7). Into the cytoplasm, one of the mature RNA strands of this duplex is incorporated into the RNA-induced silencing complex or RISC (8), and it will regulate the gene expression of its target (9-10). B: representative scheme that illustrates the *Arabidopsis* root system showing the different miRNAs that positive (green arrows) or negatively (red arrows) regulate the growth and development of the adventitious roots (AR), lateral roots (LR) or primary root (PR).

The miR164 negatively regulates the LR development by directing the cleavage of five members of the *NAM/ATAF/CUC* (*NAC*) transcription factor family, including *NAC1* (Rhoades *et al*., 2002). The over-expression of the miR164 reduces LR number and, conversely, mutants with reduced miR164 levels produce more LRs. This production is affected by the cleavage of *NAC1,* the miR164 directs the *NAC1* cleavage to down-regulate auxin signals for LR development in *Arabidopsis* (Guo *et al*., 2005). The miR167 also negatively regulates the LR development in response to nitrogen through the regulation of *ARF8*. Both miR167 and *ARF8* are specifically expressed in the pericycle and the LR cap; however, the miR167 is repressed in response to nitrogen, allowing the *ARF8* transcripts accumulate in the pericycle. Thus, the *miR167/ARF8* pathway controls nitrogen-mediated LR development (Giffor *et al*., 2008).

Besides the effects on LR development, several miRNAs-controlled genetic pathways also participate in PR growth and development. For instance, while the miR165 and miR166 participate in PR development, the miR159 and

miR396 regulate the meristem size-controlled PR growth. The miR165 and miR166 regulate the class III Homeodomain Zipper (HD-ZIP III) of transcription factors. The HD-ZIP III transcription factors are largely restricted to the vascular cylinder, and the *MIRNA165/6* are produced into the endodermis. In root endodermis and stele periphery, the regulation of the *HD-ZIP III* genes by miR165/166 is crucial to determine xylem cell types (Carlsbecker *et al*., 2010).

During the PR growth, the miR159 was identified as a key repressor. The miR159 regulates seven GAMYB-like genes, including the *MYB65*. Loss-of-function of *MIR159* genes leads to larger meristem size and consequently longer PR; and, plants expressing a miR159-resistant form of *MYB65* display longer PRs and greater cell number by increasing the cell division rates in the root meristem trough *CYCB1;1* transcription (Xue *et al*., 2017).

The miR396 is also involved in PR growth by altering the cell division and cell expansion rates. The miR396 regulates seven members of the *GROWTH-REGULATING FACTOR (GRF)* family. In plants that over-express the *MIR396B* and in the *grf1/grf2*/*grf3* triple mutant, the meristem size was larger compared with the wild type; on the other hand, plants harboring the resistant version to the miR396 cleavage for the *GRF2* and *GRF3* genes, as well as in the artificial miR396 target mimicry plants display reduction in the RMS. These changes occur by altering not only the gene expression of cell cycle-related

genes, including the *CYCB1;1* and the *CYCLIN DEPENDENT KINASE B2;1*, but also by changing the speed of the cell cycle, leading to alterations in the transition of root stem cells into transit-amplifying cells (Rodriguez *et al*., 2015); moreover, plants that over-express the *MIR396A* display opposite phenotypes on cell expansion; while the mature cortical cells reduced the length by 50%, the meristematic cortex cells increase it, indicating that miR396 has opposite effects on PR growth (Ercoli *et al*., 2016).

Another important miRNA involved in root development is the highly conserved miR156. The miR156 family, composed by eight members (*MIR156A - MIR156H)*, regulates post-transcriptionally the gene expression of most members of the *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (SPL), a plant-specific family of transcription factors present in both monocots and eudicots (Morea *et al*., 2016). The key feature of this family is the 76-amino acid SBP (*SQUAMOSA* Binding Protein) domain, which is responsible for DNA binding (Cardon *et al*., 1999). In the plant model *Arabidopsis thaliana*, there are 16 *SPL* genes, of which 10 genes contain a miR156 responsive element and, therefore, they are post-transcriptionally regulated by this miRNA (Appendix 1 A; Rhoades *et al*., 2002). The miR156-targeted *SPL* genes can be grouped into four functional clades (Appendix 1 B), being *SPL3*, *SPL10 SPL6*, and *SPL9* representative members of these clades (Guo *et al*., 2008).

The interaction between the miR156 and its targets defines a genetic regulatory pathway important for several processes as apical dominance, ovary and fruit development, male fertility, and phase transition mainly (Appendix 2; Xu *et al*., 2016; Silva *et al.,* 2014; Xing *et al.*, 2010; Chuck *et al.,* 2007). In roots, it was reported the effect of the miR156/*SPL* pathway on lateral and adventitious root production. For instance, in *Arabidopsis* plants that over-express the *MIR156A* (*p35S::MIR156A*) the LR production is higher compared with the wild-type; while reduced miR156 levels lead to fewer lateral and adventitious roots (Yu *et al*., 2015; Xu *et al*., 2016).

Among the *Arabidopsis SPLs*, the miR156-targeted *SPL3*, *SPL9*, and *SPL10* seem to be involved in repressing LR growth because transgenic plants with separate resistant version for these *SPL* genes produced fewer LR than WT with the *SPL10* playing a dominant role, and seedlings of *spl3*, *spl10* and *spl9/spl15* loss-of-function mutants all produced more lateral roots than WT under both long- and short-day conditions. These lateral root defects are attributed to LR primordia progression because r*SPL*s seedlings exhibited twice LR primordia than WT, whereas seedlings overexpressing the *MIR156* the number was reduced by 50%; additionally, the number of emerged LR in plants overexpressing the *MIR156* was higher while r*SPL*s roots showed significant lower number of emerged LR than WT (Yu *et al*., 2015).

The effect of the miR156/*SPL* pathway observed on lateral and adventitious root suggest that it also may have a possible role in PR during the progression of plant development; additionally, it is unknown whether this miR156-controlled pathway interplays with auxin and cytokinin to modulate the PR growth; thus, phenotypic, genetic and molecular mechanisms underlying the functions of miR156/*SPL* pathway in these processes deserve to be searched. For all this, we hypothesized that the miR156/*SPL* pathway regulates the PR growth by altering the root meristem size in *A. thaliana*.

2. OBJETIVES

2.1 Main objective

To evaluate the role of the miR156/*SPL* pathway during the primary root growth of *Arabidopsis thaliana*

2.2 Specific objectives

- ✓ To analyze the expression profiles of the *MIR156* and *SPL* genes during the progression of the primary root growth

- ✓ To evaluate the effect of the disruption of the miR156/*SPL* pathway balance during the primary root growth

- ✓ To analyze the auxin and cytokinin response during root growth in plants with the altered miR156/*SPL* pathway

- ✓ To identify the possible miR156-targeted *SPL* gene involved on the molecular mechanisms that regulate the primary root growth

3. MATERIAL AND METHODS

3.1 Plant material and growth conditions

All *Arabidopsis thaliana* plants used in this study were in Columbia (Col-0) ecotype. Transgenic plants expressing the *p35S::MIR156A*, miR156-sensitive (*pSPL3::sSPL3-GUS*, *pSPL6::sSPL6-GUS,* *pSPL9::sSPL9-GUS*, *pSPL10::sSPL10-GUS*) and miR156-resistant (*pSPL3::rSPL3-GUS*, *pSPL6::rSPL6-GUS,* *pSPL9::rSPL9-GUS*, *pSPL10::rSPL10-GUS*, *pSPL9::rSPL9*, *pSPL10::rSPL10*) constructs were described in Wu and Poethig (2006) and Xu *et al*. (2016). Transgenic plants harboring a target mimic against miR156 (*p35S::MIM156* or *MIM156*) were previously described (Franco-Zorrilla *et al*., 2007). *Arabidopsis* plants harboring the *pMIR156A::MIR156A-GUS* and *pMIR156C::MIR156C-GUS* constructs were described in Yang *et al*. (2013). Plants harboring the reporter constructs *pCYCLINB1;1::GUS*, *pDR5::GFP*, *pPIN1::PIN1-GFP*, and *TCSn::GFP* were described by Ferreira *et al*. (1994); Ulmasov *et al*.(1997); Su and Zhang (2009); and Zürcher *et al*. (2013) respectively. *Arabidopsis* plants harboring the reporter *pARR5::GUS* were obtained by introducing the *pARR5::GUS* construct (D'Agostino *et al*., 2000) into Col-0 plants as described by Clough and Bent (1998). At least ten independent transgenic events with similar expression patterns were obtained,

and one at T3 generation was further used. The *arr1-4* single mutant was obtained from Dello Ioio *et al* (2007), and plants with the miR156 sensor (+) and miR156 sensor (-) were obtained from Nodine and Bartel (2010).

Seeds were surface-sterilized with 1.0 % bleach for 10 min and washed five times with sterile water. Sterile seedlings were sown on sterile plates containing half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1.0 % (w/v) sugar and 0.6% (w/v) phytoagar. Plates containing the seeds were placed at 4°C for 2 days for stratification; subsequently, they were placed vertically into a growth chamber under long-day conditions (16/8 h light/dark) at 22°C.

3.2 Root growth assays and meristem size analyzes

For root growth analyzes, primary roots from seedlings at 5-, 10-, and 15-day-post-germination (dpg) were scanned and measured with *ImageJ* software (Rasband, 2012). For root growth rate (RGR), *rSPL10* and Col-0 seeds were sown on Petri dishes containing MS medium. Seedlings at 5-dpg were transfer to plates containing MS medium supplemented with 0.1 or 1.0 μM 6-Benzyladenine (6-BA), a synthetic cytokinin, and maintained for five days in a growth chamber under long-day conditions (16/8 hours light/dark) at 22°C. After four and five days of 6-BA treatment, RGR was measured using *ImageJ* software as the increment in the root length compared with 0 days after treatment. Analyzes of

RMS were performed under light microscope; the RMS was expressed as the number of cortex cells in a single file of cells extended from the quiescent center to the first elongated cell (transition zone; Dello Ioio *et al*., 2007, 2008).

3.3 RNA extraction, RT-PCR, and qRT–PCR analyzes

Total RNA was isolated using Trizol reagent (ThermoFisher) according to the manufacturer's instructions, then treated with DNase I (Invitrogen). DNase I-treated RNA (2 µg) were used to generate first-strand cDNA (Varkonyi-Gasic *et al*., 2007). qRT-PCR reactions were performed using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) and analyzed in the StepOnePlus real-time PCR system. PCR products for each primer set was subjected to melt-curve analysis, confirming the presence of only one peak on thermal dissociation generated by the thermal denaturing protocol. The threshold cycle (CT) was determined and fold-changes for each gene were calculated using the equation 2-ΔΔct (Livak and Schmittgen, 2001). Each sample was comprised of at least 50 seedlings; three biological samples with three technical replicates each were used in the analyzes. For RT-PCR expression analyzes, three biological samples were used. Expression levels were calculated relative to the housekeeping gene *ACTIN-2* or *RCH1* (a gene expressed in meristematic cells) using the ΔΔct method (Livak and Schmittgen, 2001). The primers used are listed in Appendix 3.

3.4 GUS staining and GFP analyzes

GUS staining protocol was followed according to Senecoff *et al*. (1996). GFP images were obtained from the Zeiss Epifluorescence imager D2 using specific filter for GFP analysis. For each line and treatment, at least 10 seedlings with similar expression patterns were analyzed.

3.5 Statistical analyzes

Data were represented as mean standard error (SE). Differences between independent groups were analyzed by Student, one-way ANOVA and Tukey-HSD tests for parametric data, and Mann-Whitney or Kruskal Wallis tests for non-parametric data. A p-value <0.05 was taken to indicate statistical significance.

3.6 Accession numbers

SPL2 (AT5G43270), *SPL3* (AT2G33810), *SPL4* (AT1G53160), *SPL5* (AT3G15270), *SPL6* (AT1G69170), *SPL9* (AT2G42200), *SPL10* (AT1G27370), *SPL11* (AT1G27360), *SPL13* (AT5G50570), *SPL15* (AT3G57920), *MIR156A* (AT2G25095), *MIR156C* (AT4G31877), *PIN1* (AT1G73590), *PIN3* (AT1G70940), *PIN7* (AT1G23080), *ARR1* (AT3G16857), *ARR12* (AT2G25180), *CYCLINB1;1* (AT4G37490), *ACTIN-2* (AT3G18780) and *RCH1* (Locus:1007964441).

4. RESULTS

4.1 miR156 levels decrease in primary root (PR) during the progression of plant development

In *Arabidopsis*, the *MIR156* family is composed by eight *MIR156* genes, namely *MIR156A–H* (Morea *et al.*, 2016), of which, the *MIR156A* and *MIR156C* are the major sources for the production of the mature miR156. The expression pattern of *MIR156A* and *MIR156C* genes has been studied in aerial tissues because they play central roles in the transition from the juvenile to adult phase (Yang *et al*., 2013; Xu *et al.*, 2018). During this transition, the *MIR156A* and *MIR156C* expression dramatically decrease in the aerial tissues and, as a result, the miR156-targeted *SPL* expression increase, leading to the vegetative phase change (Xu *et al*., 2016; Wu and Poethig, 2006). On PR growth, the expression of *MIR156A* and *MIR156C,* as well as the mature miR156, is poorly understood; therefore, we initially analyzed the expression profile of both *MIR156A* and *MIR156C* genes and, also, the mature miR156 in root tissues from 5-, 10- and 15-days-post-germination (dpg) seedlings.

By using transgenic plants harboring separately the *pMIR156A::MIR156A-GUS* and *pMIR156C::MIR156C-GUS* constructs, we evaluated, by GUS staining, the *MIR156A* and *MIR156C* expression. GUS staining from both *MIR156A* and *MIR156C* was detected in all PR zones of all evaluated times, although the *MIR156C* staining was broader in the meristematic and transition zones compared with *MIR156A*. As we expected, the GUS staining decreased over time for both *MIR156A* and *MIR156C* (Figure 3 A-H). To confirm these temporal expression patterns, we analyzed, by qRT-PCR, the transcript levels of *MIR156A* and *MIR156C* genes in 5-, 10-, and 15-dpg Col-0 seedlings. The transcript levels of both *MIR156A* and *MIR156C* precursors decreased over time corroborating the temporal expression observed by GUS staining (Figure 3, I-J). These results are consistent with a prominent function of these *MIR156* genes during juvenile to adult phase transition (Yang *et al*., 2013; Xu *et al*., 2018) and suggest that they may also have roles in controlling PR growth during the progression of the plant development.

The expression of mature miR156 was also confirmed using a miR156 sensor that contained a miR156 target site in the 5′ un-translated region (UTR) of a ubiquitously expressed nuclear-localized green fluorescent protein (GFP). An identical construct without the miR156 target site was used as a control (Nodine and Bartel, 2010). The miR156 sensor had reduced GFP signal throughout the meristematic and transition zones compared with the control sensor; furthermore, GFP signal, corresponding to the miR156 sensor, increased from 5- to 10-dpg,

and indicating a reduction on miR156 levels over time (Figure 3 K-N). Relative expression of mature miR156 levels, analyzed by qRT-PCR, also decreased in roots from Col-0 plants from 5- to 15-dpg (Figure 3 O). Together, these results indicate that mature miR156 is expressed and active throughout the meristematic and transition zones, and the activity decrease, as in the aerial part, during the progression of root growth.

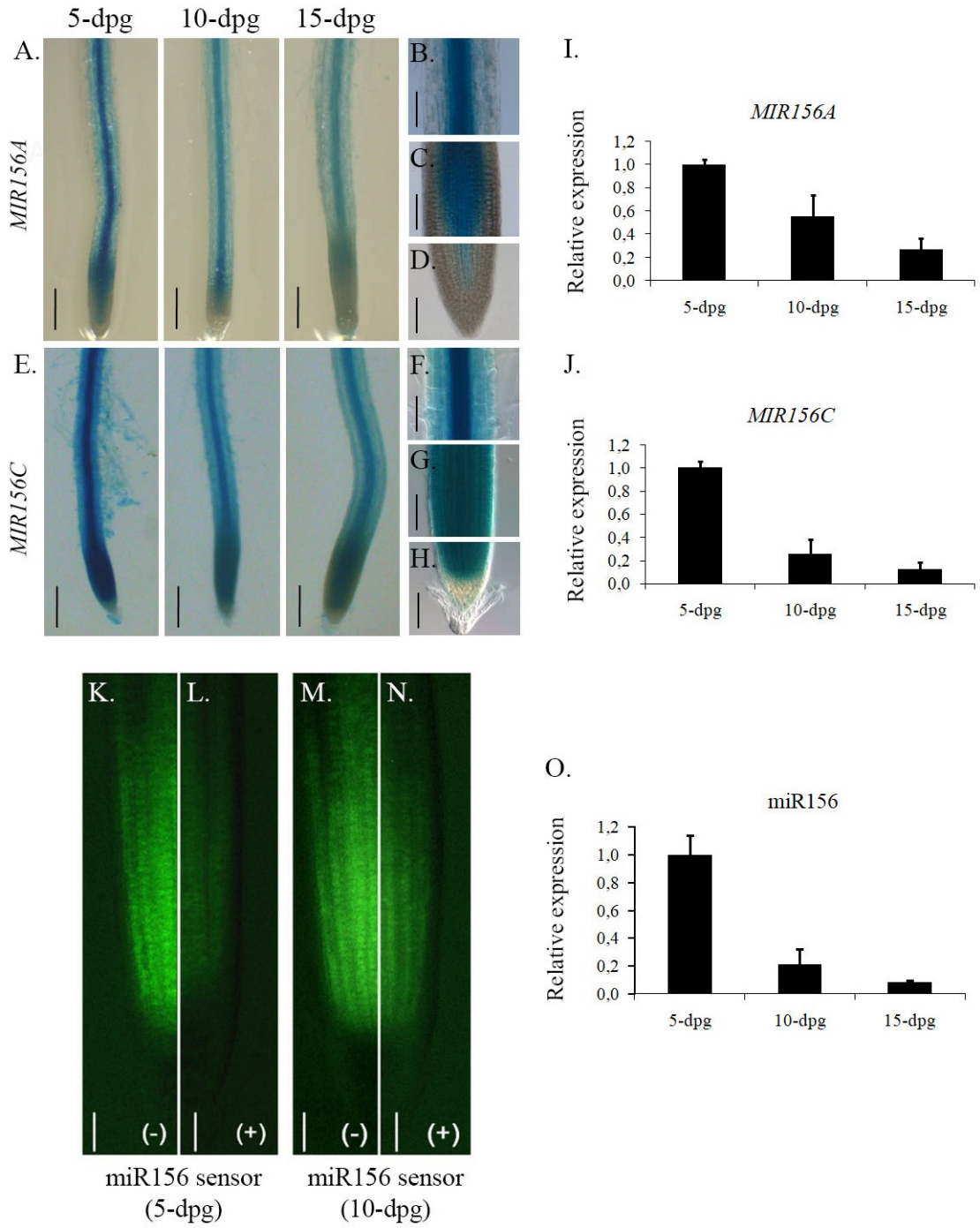

Figure 3. The *MIR156* is differentially expressed during the progression of root growth. A-D.: expression pattern by GUS staining of *MIR156A* in PR from 5-, 10- and 15-dpg seedlings (A), as well as in maturation (B) and meristematic zone (C, D). E-H.: expression pattern by GUS staining of *MIR156C* in PR from 5-, 10- and 15- dpg seedlings (E), as well as in maturation (F) and meristematic zone (G,H); scale bars: 250 µm (in A, E) and 100 µm (in B-D, F-H). I-J.: relative expression analyzes by qRT-PCR of *MIR156A* (I) and *MIR156C* (J) from roots of 5-, 10- and 15-dpg Col-0 seedlings. K-N.: epifluorescence microscope images from root tip of seedlings at 5-dpg that express the miR156 sensor constructs (GFP) with either no site (-; K) or miR156 target site (+; L), and seedlings at 10-

dpg that express the miR156 sensor constructs (GFP) with either no site ((-); M) or miR156 target site ((+); N). Images shown are representative individuals of at least 5 seedlings at each genotype expressing GPF at each developmental time. Scale bar: 50 μm. O.: relative expression analyzes by qRT-PCR of miR156 from roots of 5-, 10- and 15-dpg Col-0 seedlings. In all the qRT-PCR experiments the transcript levels were normalized to that of *ACTIN2* gene, and the expression level in 5-dpg was set as 1.0. Error bars represent standard errors (n=3 biological samples).

4.2 The *SPL3, SPL6, SPL9,* and *SPL10* display different expression patterns during primary root (PR) growth

We found that the *MIR156* expression decreases during the PR growth; accordingly, it is expected an opposing expression pattern of miR156-targeted *SPLs* genes in PR over time. The *Arabidopsis* genome contains 16 *SPL* genes, 10 of which were predicted to be post-transcriptionally regulated by the miR156 (Rhoades *et al*., 2002). The miR156-targeted *SPL* genes were grouped into four clades (Guo *et al.*, 2008); among them, *SPL3*, *SPL6*, *SPL9*, and *SPL10* are representative members of these clades, and are expressed in root tissues (Xu *et al*., 2016; Yu *et al*., 2015). To verify whether these miR156-targeted *SPL* genes are temporally regulated by the miR156 during the PR growth, we sought to obtain a more comprehensive picture of the spatiotemporal expression pattern of these representative *SPL* genes and the contribution of miR156 to this pattern in the PR growth. In this way, we analyzed the *SPL* expression, by comparing the GUS staining of 5-, 10-, and 15-dpg transgenic plants expressing a miR156-sensitive (*sSPL*), with transgenic plants expressing a miR156-resistant (*rSPL*)

version of SPL proteins tagged with ß-glucoronidase (GUS), previously described being expressed in aerial tissues (Xu *et al*., 2016).

SPL3, *SPL6*, *SPL9*, and *SPL10* displayed distinct expression patterns in PR as observed by GUS staining of *sSPL* and *rSPL* versions. GUS staining from the miR156-sensitive version of *SPL3* (*sSPL3-GUS*) was not detected in root tissues in all evaluated times; however, in the miR156-resistant version of the *SPL3* (*rSPL3-GUS*), GUS staining was observed mostly in the transition and elongation zones, especially in the stele and, as expected, it increased from 5- to 15-dpg. Interestingly, it was not observed in the meristematic zone (Figure 4 A-F); qRT-PCR data confirmed that *SPL3* transcript levels increased in root tissues of Col-0 plants from 5- to 15-dpg (Figure 4 G) suggesting a miR156-dependent regulation of *SPL3* in root tissues.

In relation to *SPL6,* we observed that, on the miR156-sensitive version (*sSPL6-GUS*), GUS staining was not detected at any developmental time point; nonetheless, on the miR156-resistant version (*rSPL6-GUS*), GUS staining was lowly but consistently detected in pericycle cells from meristematic and transition zones of 10- and 15-dpg roots (Figure 4 H-M); moreover, qRT-PCR data showed not changes in transcript levels of the *SPL6* during the progression of the PR growth (Figure 4 N).

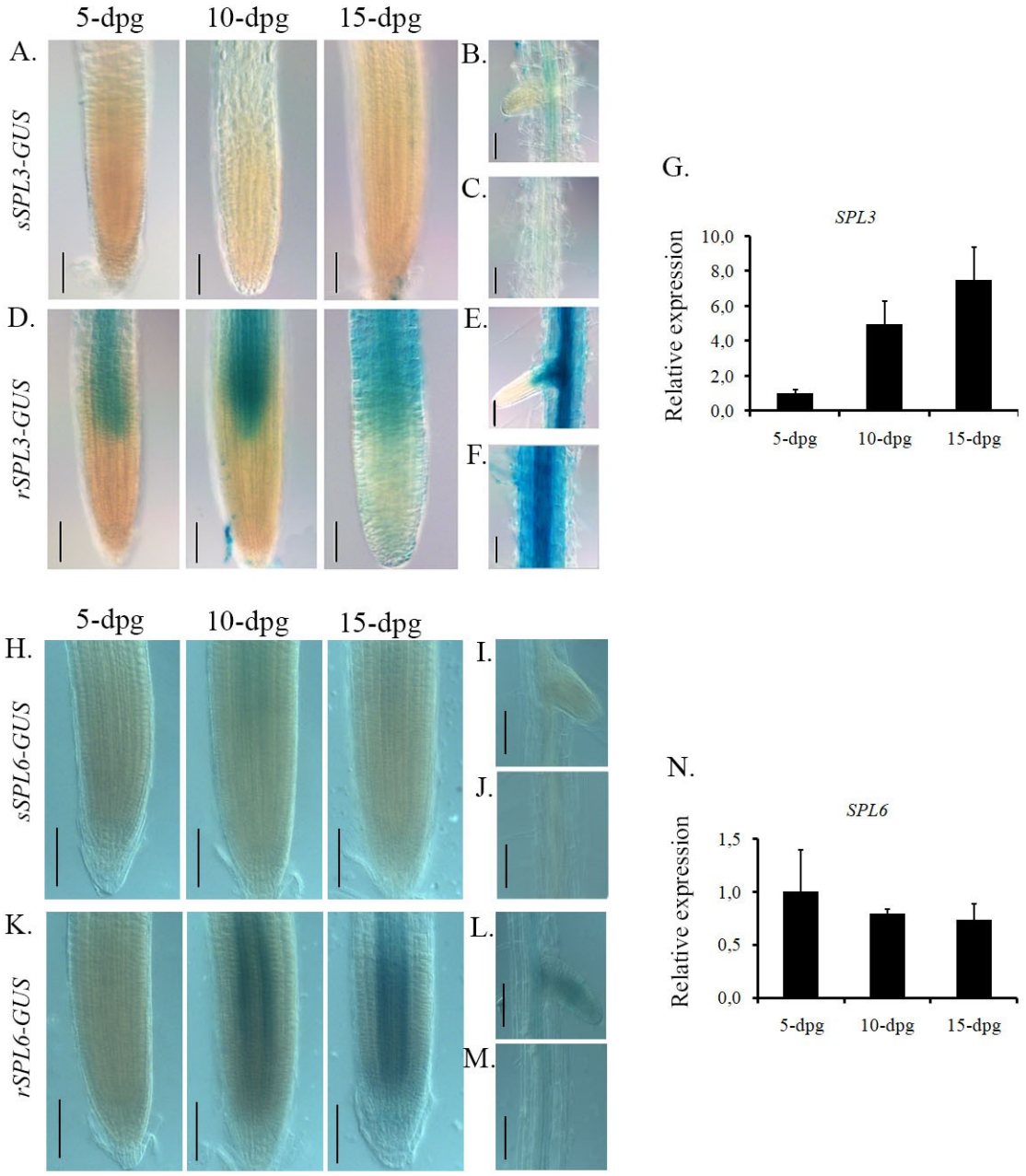

Figure 4. Gene expression profiles of the miR156-targeted *SPL3* and *SPL6* during the progression of PR growth. A-C.: expression pattern of miR156-sensitive version of *SPL3* (*sSPL3-GUS*) by GUS staining in meristematic and elongation zones (A), LR (B) and maturation zone (C). D-F.: expression pattern of miR156-resistant version of *SPL3* (*rSPL3-GUS*) by GUS staining in meristematic and elongation zones (D), LR (E) and maturation zone (F). Scale bars: 100 µm. G.: comparative expression analyzes by qRT-PCR of *SPL3* in roots of Col-0 seedlings at 5-, 10-, and 15-dpg. H-J.: expression pattern of miR156-sensitive version of *SPL6* (*sSPL6-GUS*) by GUS staining in meristematic and elongation zones (H), LR (I) and maturation zone (J). K-M.: expression pattern of miR156-resistant version of *SPL6* (*rSPL6-GUS*) by GUS staining in meristematic and elongation zones (K), LR (L) and maturation zone (M); scale bars: 100 µm. N.: comparative expression analyzes by qRT-PCR of

SPL6 in roots of 5-, 10-, and 15-dpg Col-0 seedlings. In all the qRT-PCR experiments the transcript levels were normalized to that of *ACTIN2* gene, and the expression level in 5-dpg was set as 1.0. Error bars represent standard errors (n=3 biological samples).

The miR156-sensitive version of *SPL9* (*sSPL9-GUS*) was lowly but mostly expressed in the stele of the meristematic, transition, and elongation zones. In 5-dpg seedlings, *sSPL9-GUS* was detected only in the stele at low levels; however, 10- and 15-dpg seedlings of the *sSPL9-GUS* showed GUS staining outside the stele in the elongation and maturation zones (Figure 5 A-D). In contrast, the miR156-resistant version of *SPL9* (*rSPL9-GUS*) was strongly expressed throughout the PR in all developmental times (Figure 5 E-H). Interestingly, GUS staining was reduced in both sensitive and resistant versions at 15-dpg. In roots from Col-0 seedlings at 5-, 10- and 15-dpg the analyzes by qRT-PCR showed that *SPL9* transcript levels picked at 10-dpg and it was reduced at 15-dpg roots, confirming the temporal expression patterns observed by GUS staining (Figure 5 K), suggesting a miR156-independent mechanism of *SPL9* regulation at this developmental stage.

GUS staining in roots from miR156-sensitive version of *SPL10* (*sSPL10-GUS*) was observed specifically in the meristematic zone of 10- and 15-dpg roots. On the other hand, miR156-resistant version of *SPL10-GUS* (*rSPL10-GUS*) was observed in the meristematic and transition zones in all developmental times, and it increased overtime. The qRT-PCR analyzes showed that transcript

levels of *SPL10* increased over time, confirming the temporal expression patterns observed by GUS staining (Figure 5 L-P).

Altogether, our gene expression analyzes from the representative members of the four *SPL* clades suggest that the *SPL3*, *SPL6*, *SPL9*, and *SPL10* displayed different expression patterns during the progression of the PR growth; also, the regulation of these *SPL* genes in the meristematic and transition zones is mostly miR156-dependent, and suggest a possible role of miR156/*SPL* module in the maintenance of root meristem activity and, therefore, in PR growth.

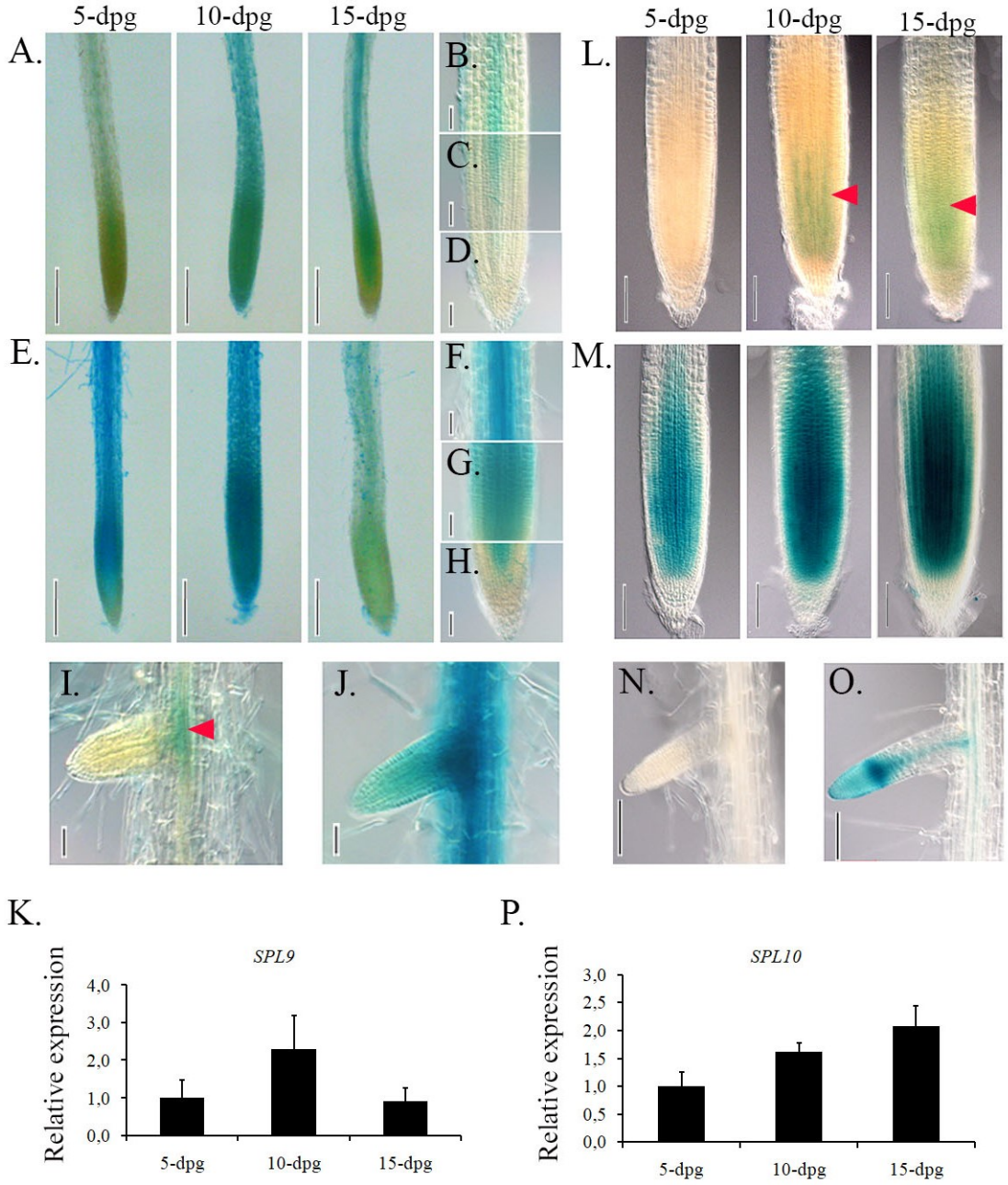

Figure 5. The miR156-targeted *SPL9* and *SPl10* are temporally regulated during the progression of PR growth. A-D.: expression pattern by GUS staining of miR156-sensitive version of *SPL9* (*sSPL9-GUS*) in PR (A–D) and LR (I; red arrowhead). E-H.: expression pattern by GUS staining of miR156-resistant version of *SPL9* (*rSPL9-GUS*) in PR (E-H) and LR (J); scale bar: 250 µm (in A, E), and 100 µm (in B-D, F-H, I-J). L, N.: expression pattern by GUS staining of miR156-sensitive version of *SPL10* (*sSPL10-GUS*) in PR (red arrowheads) and LR (N). L: expression pattern by GUS staining of miR156-resistant version of *SPL10* (*rSPL10-GUS*) in PR (M) and LR (O). Scale bar: 100 µm. Images shown are representative individuals of at least 10 GUS-stained seedlings. K, P.: relative expression analyzes by qRT-PCR of *SPL9* and *SPL10* from roots of Col-0 seedlings at 5-, 10- and 15-dpg. In all the qRT-PCR experiments the transcript

levels were normalized to that of *ACTIN2* gene, and the expression level in 5-dpg was set as 1.0. Error bars represent standard errors (n=3 biological samples).

4.3 The disruption of the miR156/*SPL* pathway balance affects primary root (PR) growth by altering the root meristem size (RMS)

Our gene expression data suggest an opposing spatiotemporal expression pattern of the miR156 and the *SPL* genes during the PR growth. It has been shown that the miR156/*SPL* pathway is involved on lateral and adventitious root development (Xu *et al.,* 2016*;* Yu *et al*., 2015), but the effect on PR growth has not been shown yet. Therefore, to gain further insight into the possible effect of the miR156/*SPL* pathway on PR growth, we initially evaluated, by RT-PCR, the expression of the miR156-targeted *SPLs* in roots tissues of 10-dpg *Arabidopsis* transgenic seedlings that express either the *MIR156A,* hereafter referred as *p35S::MIR156A* (Wu and Poethig, 2006), or a target mimic of miR156, referred as *MIM156* (Franco-Zorrilla *et al.,* 2007), both under the control of the constitutive 35S promoter of Cauliflower Mosaic Virus (*p35S*). As we expected, the transcript levels of all miR156-targeted *SPL* genes decreased in *p35S::MIR156A* and increased in *MIM156* roots (Figure 6).

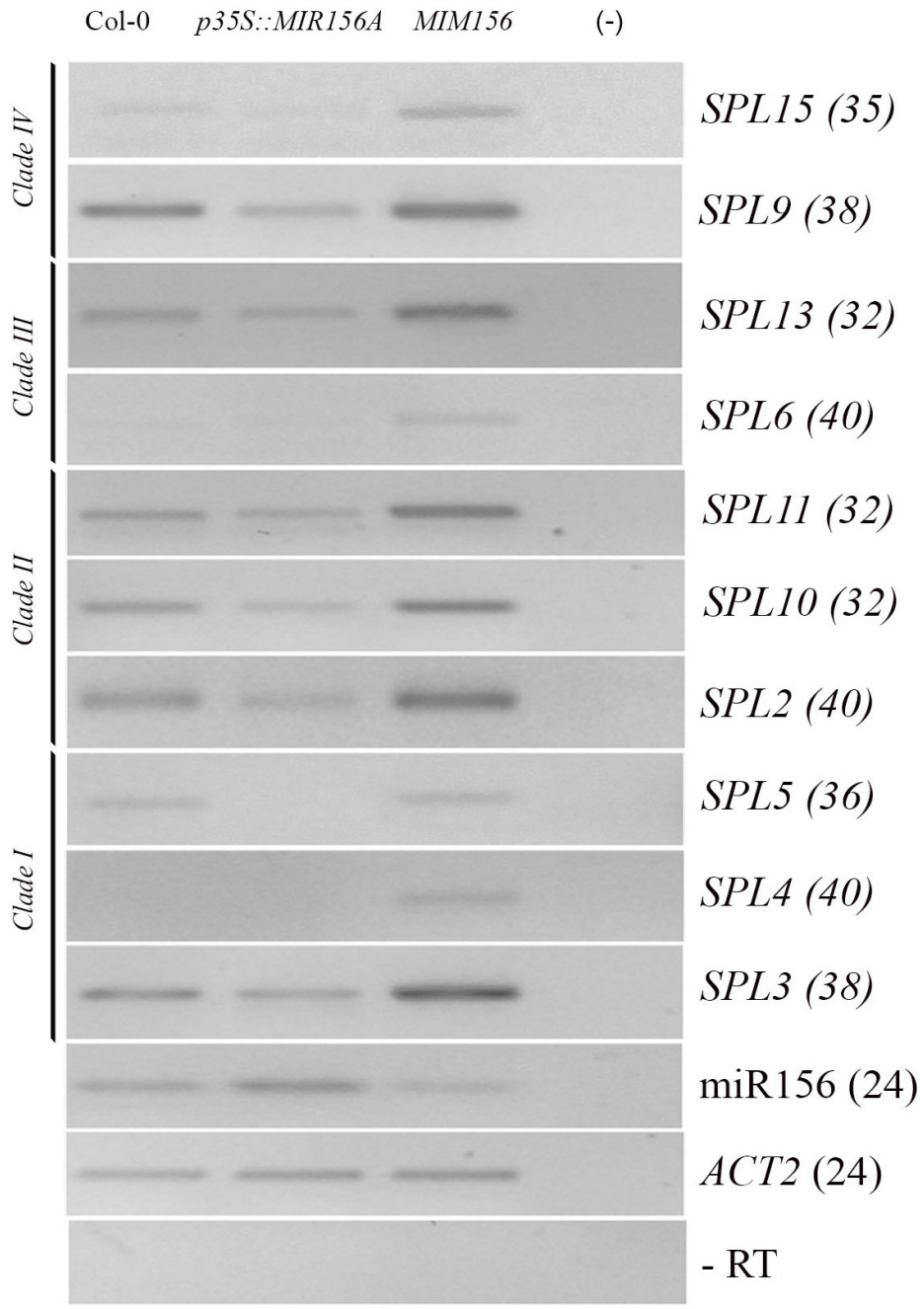

Figure 6. The miR156-targeted *SPLs* are down- and up-regulated in *p35S::MIR156A* and *MIM156* roots, respectively. Comparative expression analysis, by RT-PCR, of all miR156-targeted *SPLs* and mature miR156 in root tissues of *p35S::MIR156A*, *MIM156*, and Col-0 seedlings at 10-dpg. *SPLs* are grouped into clades according with Guo *et al*. (2008). Reactions without RT (−RT) and without cDNA (−) were used as negative controls. Numbers in parentheses indicate PCR cycles. *ACTIN2* (*ACT2*) was used as an internal control; n=3 biological samples.

Once we observed the disruption of *SPL* genes in root tissues from both *p35S::MIR156A* and *MIM156* seedlings, we decided to analyze the PR growth by measure the root length. Since root growth in these plants was reported to be not influenced by photoperiod (Yu *et al*., 2015), all experiments were performed under long-day conditions (16/8 hours light/dark). At 5-dpg, *p35S::MIR156A* seedlings displayed similar PR length than Col-0 seedlings; this may be because miR156 is already present at high levels in 5-dpg seedlings, and *SPL* genes expression is generally repressed during this developmental time phase. Interestingly, *p35S::MIR156A* seedlings at 10- and 15-dpg displayed shorter PRs; conversely, *MIM156* seedlings consistently developed longer PRs in all developmental time points (Figure 7 A-B). These findings suggest that the miR156/*SPL* pathway is involved in modulating the PR growth.

We observed that the disruption of the miR156-targeted genes results in alterations of PR growth; however, how this disruption impact the PR length is unclear. To address this question, we evaluated whether the decrease of the PR growth in *p35S::MIR156A* and the increase in the *MIM156* seedlings are mediated by the RMS. By light-microscopy analysis, we evaluated the RMS by counting the cortex-cell number in a single cell file that extends from the quiescent center (QC) to the transition zone (TZ). We found that the RMS of Col-0 seedlings was maintained constant after 5 dpg, being in agreement with Dello Ioio *et al*. (2007), whereas *p35S::MIR156A* seedlings displayed shorter

meristem at 7- and 10-dpg; in contrast, *MIM156* seedlings had larger root meristem and increased over time (Figure 7 C-D).

Our meristem size data correlates with the PR length of *p35S::MIR156A* and *MIM156* seedlings and suggest that the disruption on the miR156-targeted *SPL* genes affects the RMS by altering the cell division rates. To confirm this hypothesis, we evaluated by RT-PCR the expression analysis of the G2-M-specific *Cyclin-dependent protein kinase CYCB1;1* (*CYCB1;1*) in root tissues of Col-0, *p35S::MIR156A* and *MIM156* seedlings at 10-dpg. As we expected, the transcript levels of *CYCB1;1* were lower in *p35S::MIR156A* and higher in *MIM156* roots compared to Col-0. To confirm this expression analysis, we evaluated in 10-dpg PR meristem the GUS staining of *CYCB1;1* by transferring the *pCYCLINB1;1::GUS* construct (Ferreira *et al*., 1994) into the *p35S::MIR156A* and *MIM156* seedlings. GUS staining was lower in *p35S::MIR156A,* and more widespread from the proximal through the distal meristem in *MIM156* roots compared to Col-0 seedlings (Figure 7 E-F). Taken together, these data suggest that disruption of the miR156/*SPL* pathway balance affects primary root growth by altering the cell division-mediated RMS.

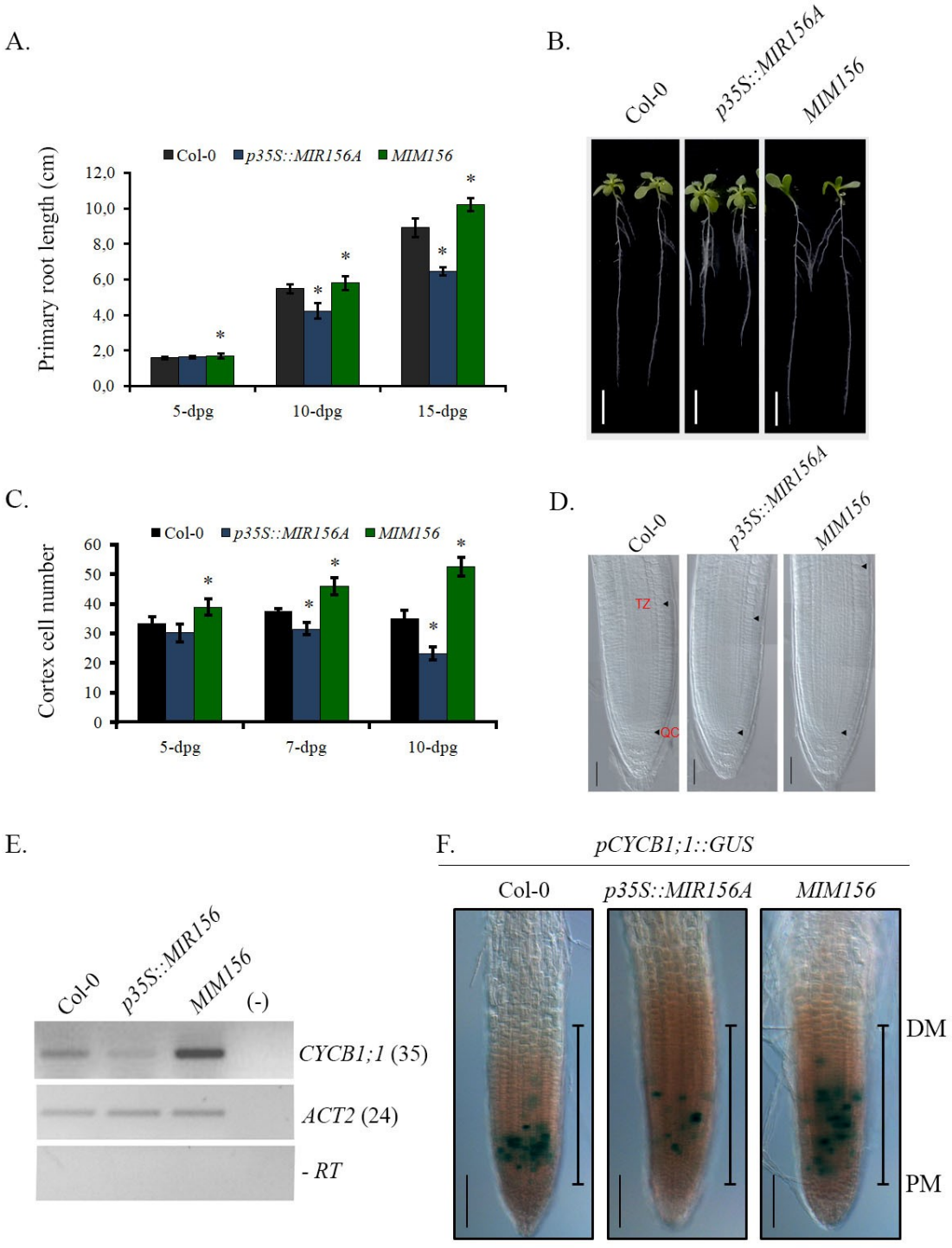

Figure 7. Disturbing the miR156/*SPL* pathway affects the primary root growth and meristem size in *Arabidopsis* by altering the cell division rates. A.: analyzes of PR length from Col-0, *p35S::MIR156A* and *MIM156* seedlings with 5-, 10-, and 15-dpg, n=50 seedlings per genotype. B.: representative pictures of root length of 10-dpg seedlings of Col-0, *p35S::MIR156A* and *MIM156;* scale bar: 1cm. C.: analyzes of RMS by counting the cortex cell number from Col-0, *p35S::MIR156A* and *MIM156* seedlings with 5-, 7-, and 10-dpg; n=20 seedlings for genotype. D.: representative pictures of PR meristem from Col-0, *p35S::MIR156A*, and *MIM156* seedlings at 10-dpg; top and bottom arrowheads

indicate the transition zone (TZ) and the quiescent center (QC) respectively; scale bar: 100 μm. E.: expression analyzes, by RT-PCR, of *CYCB1;1* in root tissues of Col-0, *p35S::MIR156A* and *MIM156* seedlings at 10-dpg; reactions without RT (−RT) and without cDNA (−) were used as negative controls; numbers in parentheses indicate PCR cycles; *ACTIN2* (*ACT2*) was used as an internal control; n=3 biological samples. F.: expression pattern by GUS staining of *CYCB1;1* in PR meristem from Col-0, *p35S::MIR156A* and *MIM156* at 10-dpg; scale bar: 100 μm; images shown are representative individuals of at least 10 GUS-stained seedlings; DM: distal meristem; PM: proximal meristem. In all experiments bars represent standard errors. Asterisk indicates significant differences (*P<0.05) according to Tukey HSD test. The experiments were repeated at least three times with similar results.

4.4 The miR156/*SPL* pathway affects the root meristem size (RMS) by altering auxin and cytokinin responses

Given that the miR156/*SPL* pathway affects the RMS, we asked whether it modulates the balance between auxin and cytokinin, a crucial component of meristem size (Dello Ioio *et al.,* 2008). Initially, we investigated the auxin response by analyzing the *pDR5::GFP* reporter in Col-0, *p35S::MIR156A*, and *MIM156* seedlings at 10-dpg. As we expected, in Col-0 background, *pDR5::GFP* is expressed in the quiescent center (QC) and columella cells. In *p35S::MIR156A* background, the *pDR5::GFP* reporter was expressed in QC and columella layers as well, but GFP signal was lower than Col-0; and in the *MIM156* background, *pDR5::GFP* was ectopically expressed, being detected also in LR cap (Figure 8 A). These data suggest that the miR156/*SPL* pathway affects the auxin response probably by altering the expression of the *PIN* auxin efflux facilitator.

Given that transporter PIN proteins maintain the auxin gradient and accumulation in the root, we next analyzed, by qRT-PCR, the expression patterns of *PIN1, PIN3*, and *PIN7,* three important genes expressed in the vascular tissue of the transition zone that help to control the RMS (Dello Ioio *et al.,* 2007). In agreement with the ectopic auxin response observed, *PIN1* and *PIN3* were up-regulated in *MIM156* roots, whereas no changes were detected in *p35S::MIR156A* roots. This observation indicates that a proper miR156:*SPL* ratio is important but not essential for *PIN* expression (Figure 8 B). By using *pPIN1::PIN1-GFP* reporter in Col-0 and *MIM156* seedlings at 10-dpg, we observed that the levels of *PIN1-GFP* protein were increased in *MIM156* roots (Figure 8 C), which is in agreement with high *PIN1* transcript levels observed in *MIM156* roots. This suggests that the miR156-targeted *SPL* de-regulation affects auxin transport in root meristem, at least partially by altering the expression of the transporter *PIN1*, and thereby affecting root meristem maintenance.

Given that auxin response was altered in plants with unbalance in the miR156-targeted *SPL*s genes, we also investigated the effects on cytokinin responses by evaluating the *ARR1, ARR12*, and *ARR5* expressions pattern in PR of 10-dpg seedlings of Col-0, *p35S::MIR156A*, and *MIM156*. The expression of *ARR1* and *ARR12*, two important genes in controlling the RMS (Dello Ioio *et al*., 2008), was evaluated by qRT-PCR, and *ARR5* expression was evaluated by GUS staining using the cytokinin-inducible reporter construct *pARR5::GUS*

(D'Agostino *et al*., 2000). Both *ARR1* and *ARR12* transcript levels were slightly higher and lower in *p35S::MIR156A* and *MIM156* roots respectively, being the expression of *ARR1* significant in *MIM156* roots (Figure 8 D); furthermore, *ARR5* expression by GUS staining was slightly higher in *p35S::MIR156A* and lower in *MIM156* seedlings (Figure 8 E). Altogether, our analyzes indicate that *SPL* de-regulation leads to lower cytokinin responses and, therefore, impacts on meristem size-controlled PR growth.

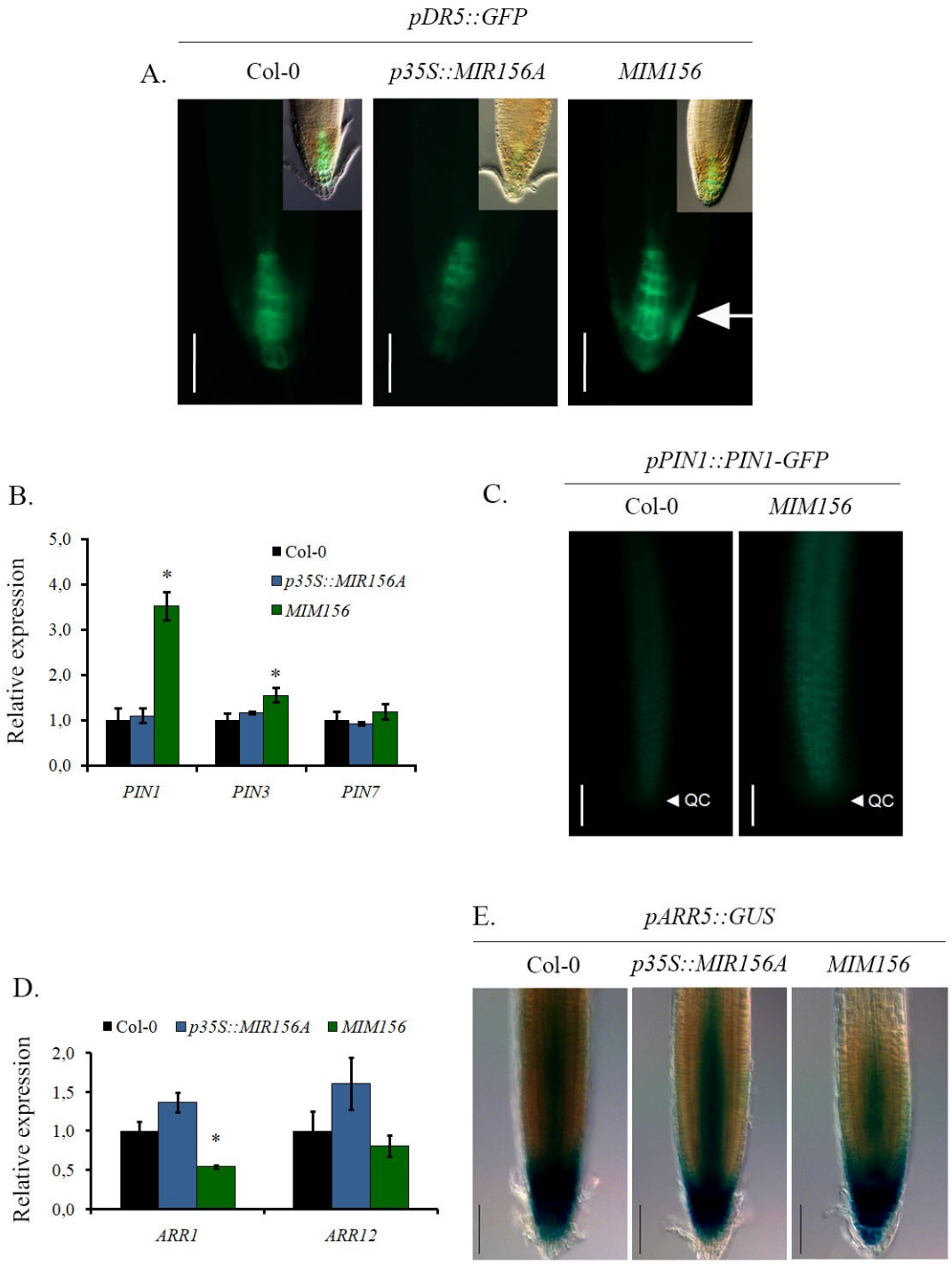

Figure 8. The auxin and cytokinin responses are disturbed in primary root from *p35S::MIR156A* and *MIM156* seedlings. A.: auxin response analysis by GFP in Col-0, *p35S::MIR156A*, and *MIM156* seedlings at 10-dpg using the *pDR5::GFP* reporter. Representative epi-fluorescence microscope pictures from meristem tip. Scale bar: 100 µm. Insets represent merged images. Arrow indicates ectopic *GFP* expression in LR cap. Images shown are representative individuals of at least 15 seedlings. B.: relative expression analyzes by qRT-PCR of *PIN1, PIN3* and *PIN7* genes from roots of Col-0, *p35S::MIR156A*, and *MIM156* seedlings at 10-dpg. C.: auxin transport analysis by GFP signals from 10-dpg Col-0 and *MIM156* seedlings, using the *pPIN1::PIN1:GFP* reporter.

Representative epi-fluorescence microscope images of PR meristem; scale bar: 100 μm; arrowheads indicate the quiescent center (QC); images shown are representative individuals of at least 10 seedlings. D.: relative expression analysis by qRT-PCR of *ARR1* and *ARR12* from 10-dpg roots of Col-0, *p35S::MIR156A*, and *MIM156* seedlings. E.: expression pattern by GUS staining of *ARR5* in root meristem from 10-dpg Col-0, *p35S::MIR156A* and *MIM156* seedlings; scale bar: 100 μm; images shown are representative individuals of at least 10 GUS-stained seedlings. In all the qRT-PCR experiments the transcript levels were normalized to that of *ACTIN2* gene, and the expression level in Col-0 was set as 1.0. Error bars represent standard errors (n=3 biological samples). Asterisk indicates significant difference (*P<0.05) according to Mann Whitney test. The experiments were repeated at least three times with similar results.

4.5 The de-regulation of the miR156-targeted *SPL10* increases the primary root (PR) length

So far, our data suggests that the miR156/*SPL* pathway impacts the PR growth, with down-regulation of all the miR156-targeted *SPL* genes reducing the PR length, and the de-regulation increasing it. These data suggest that at least one *SPL* gene is involved in the regulation of the PR length in *Arabidopsis.* To test this hypothesis, we decided to evaluate which miR156-targeted *SPL* gene may be involved in controlling the PR length of *Arabidopsis.* The *SPL* family is composed by 16 members; 10 out of those are cleaved by the miR156-directed AGO protein, and they can be grouped into four functional clades, being *SPL3*, *SPL6*, *SPL9* and *SPL10* representative members of these clades (Guo *et al*., 2008). It was observed that single mutants of those *SPL* genes have little or no effect on aerial parts of the plant body; however, their de-regulation, by introducing a miR156-resistant version of specific *SPL* genes under the control of the native promoter, can lead to strong phenotype (Xu *et al*., 2016).

In the light of this information, we initially evaluated the PR length in *Arabidopsis* transgenic plants that, separately, express a miR156-resistant version of *SPL3* (*rSPL3*), *SPL6* (*rSPL6*), *SPL9* (*rSPL9*) and *SPL10* (*rSPL10*) genes, all of them under the control of the native promoter. These transgenic plants were previously described (Xu *et al*., 2016; Wu *et al.,* 2009). The PR length of the *rSPL3*, *rSPL6*, and *rSPL9* transgenic plants displayed similar PR length than Col-0 seedlings at 5-, 10, and 15-dpg; nevertheless, the *rSPL10* transgenic plants consistently developed longer PR in all developmental time points (Figure 9 A-B). These findings suggest that the de-regulation of the *SPL10* may be involved in modulating the PR growth.

Our data shows that the de-regulation of the *SPL10* gene correlates to longer PR; this phenotype agrees with the *MIM156* seedlings phenotype, previously shown, in which all miR156-targeted *SPL* genes are de-regulated and, also, it is opposite to root phenotype of *p35S::MIR156A* plants, in which all the miR156-targeted *SPL* genes are down-regulated and the PR is shorter compared to Col-0. In order to investigate the effect of the loss-of-function of *SPL10* on the PR length, we decided to analyze the PR on the *spl10* single mutant; nonetheless, the available *spl10* mutant (Nodine and Bartel, 2010) contains a T-DNA insertion in the first exon of *SPL10*, but this insertion does not reduce the *SPL10* transcript, and does not have an obvious phenotype in the aerial part of the plant (Xu *et al*., 2016). Thus, we evaluated the root growth in *SPL10-RNAi* plants,

which contain low levels of *SPL10* transcripts (Nodine and Bartel, 2010). *SPL10-RNAi* seedlings did not differ from Col-0 in terms of PR length in all developmental time points (Figure 9 C-D), suggesting a functional redundancy with other *SPL* members of the same clade.

Because of the knock-down of only *SPL10* is not sufficient to reduce PR length as observed in *p35S::MIR156A* roots, we wanted to further substantiate that the down-regulation of *SPL10* is partially responsible for the PR length reduction in *p35S::MIR156A* seedlings; thus, we crossed *rSPL10* plants with *Arabidopsis* plants harboring *p35S::MIR156A*. The de-regulation of *SPL10* in the *p35S::MIR156A* seedlings was sufficient to rescue PR growth at Col-0 levels in the F1 *p35S::MIR156A;rSPL10* seedlings (Figure 9 E-F). Together, our observations confirm the importance of the miR156:*SPL* ratio in the control of PR growth.

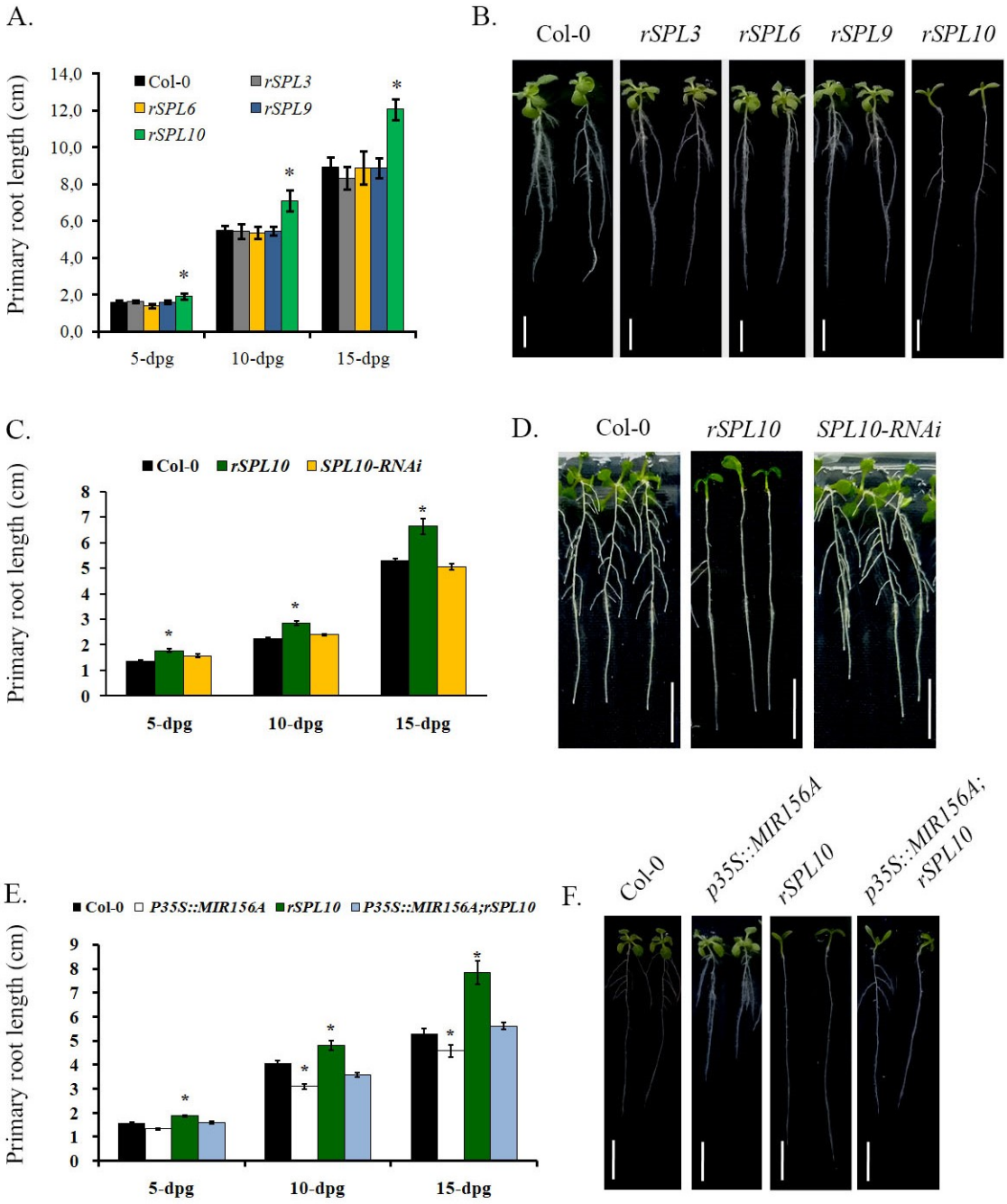

Figure 9. The PR length is increased by the de-regulation of *SPL10*. A.: analyzes of PR length from Col-0, *rSPL3*, *rSPL6*, *rSPL9*, and *rSPL10* transgenic seedlings at 5-, 10-, and 15-dpg, n=50 seedlings per genotype. B.: representative pictures of root length of 10-dpg Col-0, *rSPL3*, *rSPL6*, *rSPL9*, and *rSPL10* seedlings*;* scale bar: 1cm. C.: analyzes of PR length from Col-0, *rSPL10* and *SPL10-RNAi* seedlings at 5-, 10-, and 15-dpg, n=30 seedlings per genotype. D.: representative pictures of root length of 10-dpg seedlings from Col-0, *rSPL10* and *SPL10-RNAi;* scale bar: 1cm. E.: analysis of PR length from Col-0, *p35S::MIR156A, rSPL10* and the double transgenic *p35S::MIR156A;rSPL10,* at 5-, 10-, and 15-dpg, n=30 seedlings per genotype. F.: representative pictures of root length of 10-dpg seedlings from Col-0, *p35S::MIR156A, rSPL10* and the double-transgenic *p35S::MIR156A;rSPL10;* scale bar: 1cm. In all experiments

error bars represent standard errors. Asterisk indicates significant differences (*P<0.05) according to Tukey HSD test. The experiments were repeated at least three times with similar results.

4.6 The de-regulation of the miR156-targeted *SPL10* increases the root meristem size (RMS) by promoting cell cycle activity

To investigate if the de-regulation of the *SPL10* affects the PR growth by altering the RMS, we evaluated the RMS by counting, in a light-microscopy, the number of cell cortex in a single cell file of the cortex which extends from the quiescent center (QC) to the transition zone (TZ), from PR of 10-dpg *rSPL3*, *rSPL6*, *rSPL9*, and *rSPL10* transgenic plants. We found that the *rSPL10* plants, as we expected, displayed larger RMS while *rSPL3*, *rSPL6*, and *rSPL9* transgenic lines did not changed compare to Col-0 (Figure 10 A-B). These data suggest that the de-regulation of the *SPL10* increases the RMS and, therefore, the PR growth in *Arabidopsis*.

The *rSPL10* seedlings consistently displayed more cortex cells in the meristematic zone, suggesting that *SPL10* might increase the cell division rates on meristematic cells. To test this hypothesis, we performed quantitative reverse-transcriptase (qRT-PCR) expression analysis of the G2-M-specific *Cyclin-dependent protein kinase CYCB1;1* in 10 dpg Col-0, *rSPL9* and *rSPL10* roots. Although *SPL9* is expressed in the meristematic zone and its expression changes during the progression of the root growth, *rSPL9* seedlings did not show

significant changes in PR growth or meristem size. Therefore, we included 10-dpg *rSPL9* roots in our expression analyzes along with 10-dpg Col-0 and *rSPL10* to determine whether possible changes in *CYCLINB1;1* expression is due to the *SPL10* de-regulation and not to stochastic changes in *SPL* expression.

Moreover, to overcome the difference in root length between samples, we normalized *CYCLINB1;1* expression level to *RCH1* transcripts, a gene specifically expressed in meristematic cells (Casamitjana-Martinez *et al*., 2003). *CYCB1;1* was significantly up-regulated in *rSPL10*, but not in *rSPL9* roots (Figure 10 C). To visualize *CYCB1;1* expression observed in *rSPL10* roots, we transfer the *pCYCLINB1;1::GUS* construct (Ferreira *et al*., 1994) into *rSPL10* plants. Indeed, an increase in the expression levels of the *pCYCB1;1::GUS* reporter was observed in *rSPL10* root tips (Figure 10 D). It is possible that the alterations in *CYCB1;1* expression in *rSPL10* seedlings reflect a faster progression and/or an earlier G2/M transition of the cell cycle, thus, suggesting that miR156-targeted *SPL10* may be involved in cell cycle control.

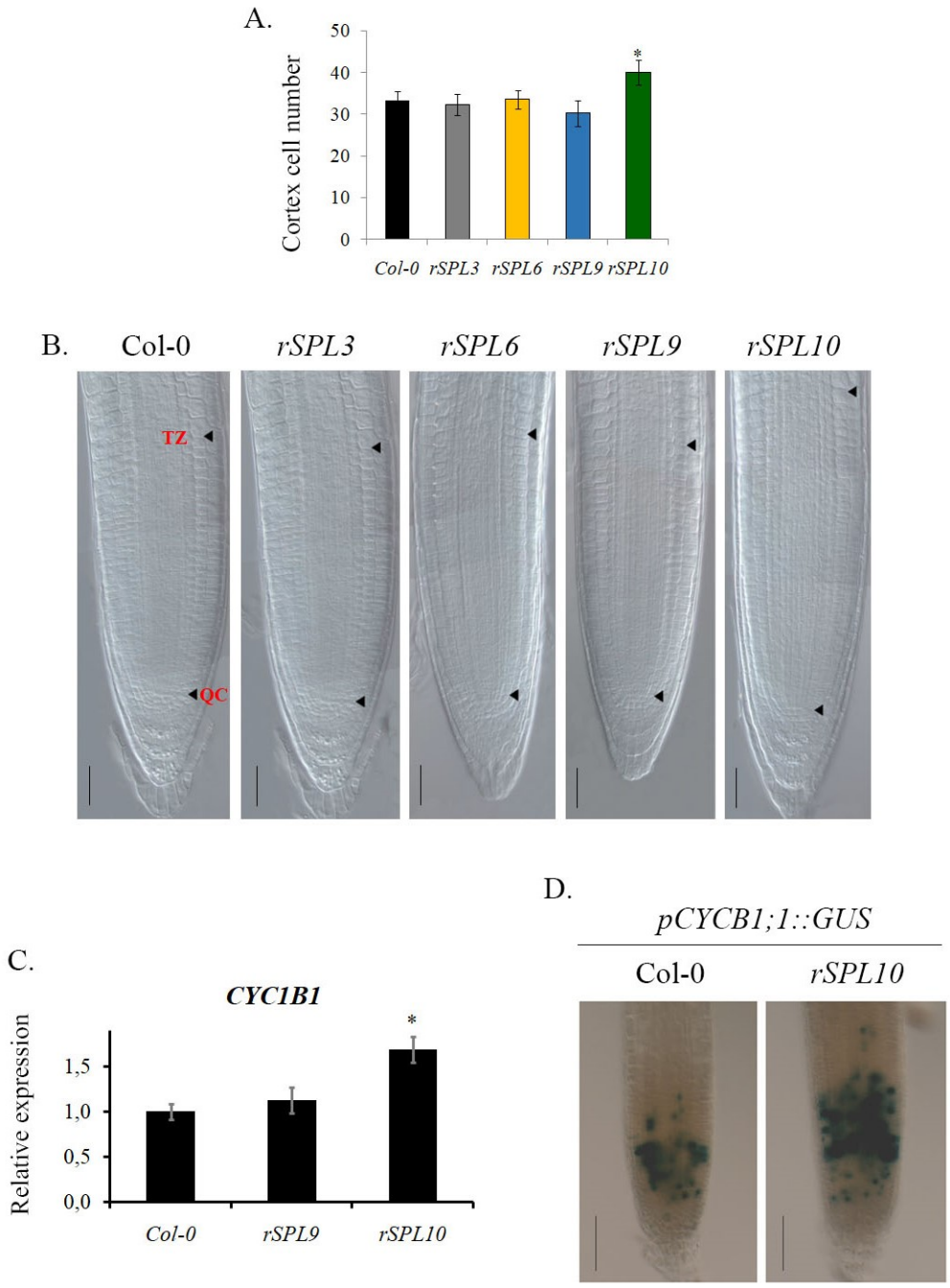

Figure 10. The cell cycle activity in root meristem may be regulated by the *SPL10*. A.: analyzes of RMS by counting the cortex cell number from Col-0, *rSPL3*, *rSPL6*, *rSPL9*, and *rSPL10* seedlings at 10-dpg; n=20 seedlings for genotype. B.: representative pictures of PR meristem from Col-0, *rSPL3, rSPL6, rSPL9,* and *rSPL10* seedlings at 10-dpg; Top and bottom arrowheads indicate the transition zone (TZ) and the quiescent center (QC), respectively; scale bar: 100 µm. C.: relative expression analysis by qRT-PCR of *CYCB1;1* from roots of 10-dpg Col-0, *rSPL9,* and *rSPL10* seedlings; the transcript levels were normalized to that of *RCH1* gene, and the expression level in Col-0 was set as 1.0; (n=3 biological samples). D.: expression pattern by GUS staining of *CYCB1;1* in root meristem from Col-0, and *rSPL10* at 10-dpg; scale bar=100 µm; images shown

are representative individuals of at least 12 GUS-stained seedlings. In all experiments error bars represent standard errors. Asterisk indicates significant difference (*P<0.05) according to Tukey HSD (in A) or Mann Whitney test (in C).

4.7 The de-regulation of the *SPL10* increases the root meristem size (RMS) by altering cytokinin responses

Given that the de-regulation of the *SPL10* increases the RMS by altering the cell cycle activity, we then asked if the *SPL10* also affects the balance between auxin and cytokinin. This appropriate balance, according to Dello Ioio *et al.* (2008), controls the RMS by the antagonistic effects of auxin which mediates cell division, and cytokinin which mediates cell differentiation. To further study this possible interplay between the de-regulation of the *SPL10* and the auxin/cytokinin ratio, we investigated the auxin response by analyzing the relative expression of the *PIN1, PIN3,* and *PIN7* genes, and the cytokinin response by analyzing the *ARR1* and *ARR12* gene expressions.

PIN1, PIN3 and *PIN7* expression did not change in r*SPL10* roots compared to Col-0 (Figure 11 A); this observation suggests that particular *SPLs* in addition to *SPL10* might be involved in the regulation of these *PINs* since no significant change in their transcript levels was observed in *rSPL10* roots. On the other hand, the cytokinin response, assessed by the *ARR*s expression, shows that *ARR1* expression in root tissues was significantly lower in r*SPL10* compared to

Col-0 (Figure 11 B). This finding indicates that *SPL10* de-regulation leads to lower cytokinin responses by affecting *ARR1* transcript levels.

To substantiate the role of the *SPL10* in modulating cytokinin responses in roots, we compared the responses to this hormone in Col-0 and *rSPL10* roots by using the *Two Component signaling Sensor new* (*TCSn*)*::GFP* reporter, which reflects the transcriptional activity of *type-B ARRs* (Zürcher *et al*., 2013). We also analyzed the widely used *pARR5::GUS* cytokinin reporter (D'Agostino *et al*., 2000). In Col-0 roots, *GFP* expression was observed in the columella cells of the root meristem and into the vasculature, whereas reduction in GFP levels was observed in similar cell types of *rSPL10* roots (Figure 11 C). Likewise, reduction of *pARR5::GUS* activity was detected in *rSPL10* roots when comparing with Col-0 (Figure 11 D). These results suggest that low cytokinin response in *rSPL10* may be responsible for the unbalance auxin:cytokinin functions inducing higher cell proliferation rate in the root meristem.

Based on the low cytokinin response observed in *rSPL10* seedlings, and on the reduction of the RMS and overall PR growth by exogenous application of cytokinin (Dello Ioio *et al*., 2007), we hypothesized that the low response to cytokinin observed in *rSPL10* roots may decrease exogenous cytokinin effects. To test this hypothesis, we treated Col-0 and *rSPL10* roots with two concentrations of 6-Benzyladenine (6-BA), a synthetic cytokinin (Dello Ioio *et*

al., 2007), and analyzed the root growth rate (RGR) at four and five days post-treatment (dpt). Interestingly, RGR was similar between Col-0 and *rSPL10* roots growing in control MS medium at 4- and 5 dpt. At low 6-BA concentration (0.1 µM), RGR was lower in Col-0 than in *rSPL10* roots in both 4 and 5 dpt, suggesting that *rSPL10* seedlings were less sensitive to this cytokinin treatment. At higher 6-BA concentration (1.0 µM), the differences between Col-0 and *rSPL10* responses in terms of RGR were less pronounced, but *rSPL10* roots were still less affected by 6-BA treatment than Col-0 roots (Figure 11 E; Appendix 4). Together, these observations agree with the idea that *SPL10* de-regulation leads to lower cytokinin responses in roots.

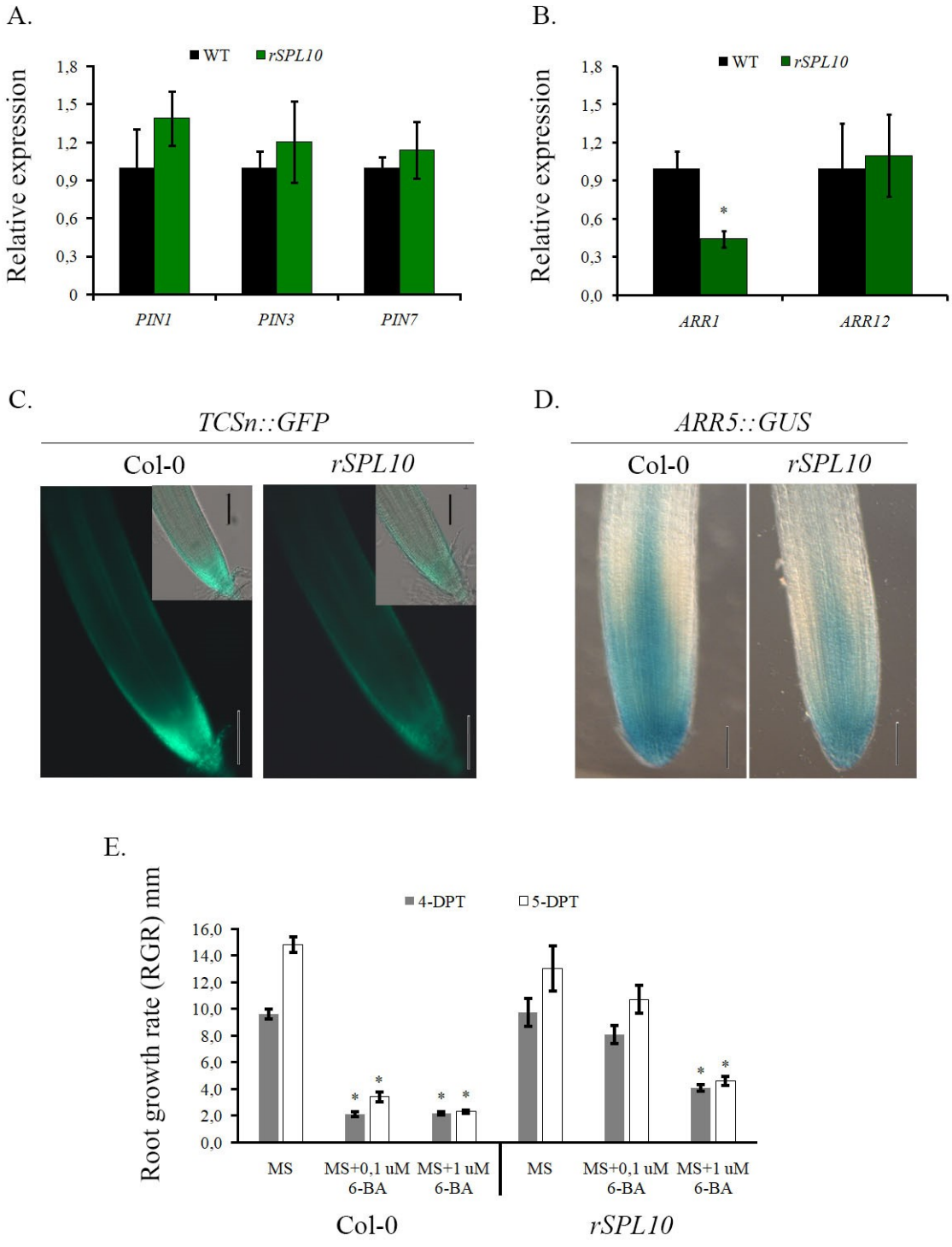

Figure 11. The de-regulation of the miR156-targeted *SPL10* attenuates cytokinin responses in primary root. A.: relative expression analysis by qRT-PCR of the *PIN1, PIN3* and *PIN7* genes from roots of Col-0 and *rSPL10* seedlings at 10-dpg. B.: relative expression analysis by qRT-PCR of the *ARR1* and *ARR12* genes from roots of Col-0 and *rSPL10* seedlings at 10-dpg. C.: analysis of cytokinin responses by *TCSn::GFP* reporter from roots of Col-0 and *rSPL10* seedlings at 10-dpg; representative epifluorescence microscope images of PR meristem; scale bar: 100 µm. Insets represent merged images. D.: GUS

staining of *ARR5* in roots from Col-0 and *rSPL10* seedlings at 10-dpg using the *pARR5::GUS* reporter; scale bar: 100 μm. E.: analysis of root growth rate (RGR) of Col-0 and *rSPL10* seedlings growing in MS medium with or without 6-Benzyladenine (6-BA); RGR was evaluated at four and five days post treatment (DPT; n= 45 seedlings per genotype per treatment); asterisks indicate a significant difference compared to reference sample (MS medium) according to Student's t test (**P*<0.01); the experiment was repeated three times with similar results. In all the qRT-PCR experiments the transcript levels were normalized to that of *ACTIN2* gene. The expression level in Col-0 was set as 1.0. Error bars represent standard errors (n = 3 biological samples). Asterisks indicate a significant difference (*P<0.05) compared to reference sample according to Mann-Whitney test.

5. DISCUSSION

5.1 The miR156 is active in primary root (PR) and its levels decrease overtime as in the aerial part

The miR156 and its targets, most members of the *SPL* family, are the master regulators of phase change in both monocots and eudicots, and it has been confirmed by a growing number of reports, mostly focused on the aerial organs, such as shoot maturation, plastochron length and organ size, heteroblasty, vegetative phase change, and floral induction, flower and fruit development (Schwarz *et al.*, 2008; Wang *et al.*, 2008; Usami *et al.*, 2009; Wu *et al.*, 2009; Yu *et al.*, 2010; Silva *et al.*, 2014; Silva *et al.*, 2018). In roots, it was demonstrated that the miR156/*SPL* pathway also participates in the development of the root tissues by altering the number of lateral and adventitious roots in *Arabidopsis* (Yu *et al*., 2015; Xu *et al*., 2016). In this work, we show not only the temporal expression profiles of the mature miR156 in root tissues, but also, the spatial expression patterns of both the mature miR156 and the *MIR156A* and *MIR156C* genes, two major sources for the production of the mature miR156 in the aerial tissues. Our data suggest that those genes also participate in the mature miR156 production in root tissues.

Our analyzes show that the *MIR156A* and *MIR156C* as well as the mature miR156 level, decrease in root tissues during the progression of plant development, as in the aerial part of the plant (Yang *et al*., 2013; Xu *et al.*, 2018), suggesting that the miR156 is also a temporal regulator in root tissues. Importantly, miR156 activity was detected in the elongation and meristematic zones, suggesting its role in regulating the PR growth and development overtime.

5.2 Most of the miR156-targeted *SPL*s are post-transcriptionally regulated in root tissues and their expression increase overtime

The miR156 levels dramatically decrease in the aerial tissues and, consequently, the miR156-targeted *SPL* expression increase, allowing the vegetative phase change (Xu *et al*., 2016; Wu and Poethig, 2006). We show that the miR156 levels also decrease overtime in root tissues and, consequently, lead to increase the expression of most miR156-targeted *SPL* genes during the progression of the root growth; however, our data show that the *SPL6* remains constant over time; additionally, *SPL9* expression dramatically decreases in 15-dpg roots. It was reported that the *SPL9*, a miR156 target, positively regulates *MIR156* genes (Yu *et al*., 2015); thus, our data suggests that the *SPL9* is temporally regulated, not only by the mature miR156, but also by a miR156-independent mechanism to ensure low levels of the miR156 overtime and, therefore, leading to phase transition.

It was reported an increased expression of the *SPL* genes on the aerial part (Xu *et al*., 2016) and in root tissues (Yu *et al.,* 2015). Here, we show not only the temporal expression, but also the spatial expression patterns of the representative *SPL*s, from the four functional clades (Guo *et al*., 2008), in the whole PR with special emphasis on meristematic zone. We included analyzes of 5-dpg roots, an important time point regarding root meristem size establishment. At this time, in wild type plants the meristem size is established and it maintains constant over-time (Dello Ioio *et al.,* 2007). The spatial expression patterns of the representative *SPL* genes suggest that the miR156-targeted *SPLs* might have a role in delineating the region between elongating and mature root cells since *SPLs* have higher and lower expression in the elongation and maturation root zones, respectively, whilst miR156 show the opposite expression pattern.

5.3 Disruption of the miR156:*SPL* balance affects primary root (PR) growth by altering the root meristem size (RMS)

Modifying the *SPL* transcript levels, either by over-expression of the *MIR156A* gene, in *p35S::AMIR156A* seedlings, or by reducing the available mature miR156, in *MIM156* plants, leads to strong phenotypes on the aerial part of the plant as changes in format and number of leaves, plastochron length, apical dominance, etc (Wu and Poethig, 2006; Franco-Zorrilla *et al*., 2007). In root tissues, alteration in the transcript levels of the *SPL* genes also leads to changes in the phenotype such as alterations in the number of the lateral and adventitious roots (Yu *et al*., 2015; Xu *et al.,* 2016). Here, we show that the over-expression

of the *MIR156A* gene leads to shorter RMS and, consequently, shorter PR. On the other hand, the de-regulation of the *SPL* genes brings, as a consequence, larger RMS and, therefore, longer PR. Thus, our data indicate that the miR156/*SPL* pathway has much broader effect in *Arabidopsis* root system than previously reported.

The PR length is associated with the RMS, and the latter with cell division rates. In Col-0, for instance, the RMS is fixed in the fifth day post-germination; during the RMS establishment, several factors participate including the auxin and cytokinin balance and some miRNAs. For instance, while the miR396 positively impacts the cell division (Rodriguez *et al*., 2015), the miR159 negatively impacts the cell division rates on root meristem (Xue *et al*., 2017). Our data show that the miR156 is a negative regulator of the RMS because the over-expression of the *MIR156A* gene leads to small RMS and, in the opposite way the de-regulation of the miR156-targeted *SPL* genes increases the RMS. Thus, our data contributes to uncover the miRNAs-associated mechanisms by which the cell division-mediated RMS is established.

5.4 Disruption of the miR156:*SPL* balance affects the auxin and cytokinin responses in primary root (PR)

The RMS is established by different factors, especially by the balance between the antagonistic effects of auxin mediating cell division, and cytokinin

mediating cell differentiation (Dello Ioio *et al.,* 2008). The interplay between phytohormones, especially auxin and cytokinin, and miRNAs during the establishment of the RMS is poorly understood. A recent study showed that the *PIN1* expression between WT and transgenic plants harboring the *pMYB65:mMYB65,* was not greatly altered (Xue *et al*., 2017). Here, we evaluated not only the *PIN1* expression by also the *PIN3* and *PIN7* genes, and overall the auxin response by visualizing the *pDR5::GFP* in plants with disruption of the *SPL* expression. Both *PIN1* and *PIN3* gene expression were significantly up regulated in *MIM* plants; thus, our study constitutes an evidence of the interaction between miRNAs and phytohormones.

Regarding the interplay between miRNAs and the RMS-associated cytokinin responsive genes, we did not found information available; thus, we decide to evaluate the cytokinin responses by analyzing the *ARR1, ARR12* and *ARR5* cytokinin responsive genes. Our data suggest that the cytokinin responses are affected by the de-regulation of the SPLs genes. It was shown that *ARR1* is capable alone of controlling the RMS (Dello Ioio *et al*., 2008) because of the loss-of-function *arr1-4* mutant display larger PR and larger root meristem (Dello Ioio *et al.,* 2007) and plants over-expressing the *ARR1* gene display shorter PR (Sakai *et al*., 2001). Based on those results, we think that the reduction of the *ARR1* levels in *MIM156* plants is associated with the increase in the RMS; thus, our work would constitute the first work that shows the interplay between the

miR156/*SPL* pathway and the auxin/cytokinin responsive genes during the establishment of the RMS in *Arabidopsis*.

5.5 The de-regulation of the *SPL10* leads to larger root meristem size (RMS) and longer primary root (PR)

The *Arabidopsis* genome contains 10 miR156-targeted *SPL* genes, and it was shown that most of them are expressed in root tissues (Yu *et al*., 2015; Xu *et al.,* 2016). Here we show that all the miR156-targeted *SPL* genes are differentially expressed on PR and their expression changes during the progression of the PR, with the increasing through time being the central characteristic. Although, the loss-of-function of individual miR156-targeted *SPL* genes does not affect the phenotype on the aerial tissues (Xu *et al*., 2016), the de-regulation of specific miR156-targeted *SPL* genes leads to strong phenotype (Wu *et al*., 2009). Thus, it is expected no alter phenotype in root tissues by the loss-of-function on specific *SPL* genes, and strong phenotype by the de-regulation of specific miR156-targeted *SPL* genes.

Yu *et al*. (2015) showed that transgenic plants with the resistant version of the *SPL3*, *SPL9*, and *SPL10* to the miR156 cleavage display lower lateral root growth; with *SPL10* playing a dominant role; also, Xu *et al*. (2016) showed that the de-regulation of several *SPL* genes affected the adventitious root production. The information about the de-regulation of the *SPL* genes on PR is unknown.

Here, we show that de-regulation of the *SPL10* positively impacts the RMS and consequently the PR length in *Arabidopsis*.

5.6 The de-regulation of the *SPL10* enhances the cell cycle activity in primary root (PR)

The maintenance of normal levels of *SPL* expression during early stages of root growth may be crucial for the correct balance between the rates of cell division and differentiation. This correct balance in turn determines the size of the root meristem and the root growth (Dello Ioio *et al*., 2007). In our study, the miR156-over-expressing and miR156-target mimicry roots displayed lower and higher meristem cell number, respectively, suggesting that a correct threshold-dependent repression of the *SPL* expression by miR156 contributes to regulate the balance between cell proliferation and differentiation.

In *Arabidopsis* leaves, heteroblasty-promoting *SPL* genes *SPL3*, *-4*, *-5* and *-15* increase cell number and reduce cell size (Usami *et al.*, 2009). Here, we show that plants expressing the miR156-resistant version of *SPL10* (*rSPL10*) have increased RMS by promoting directly or indirectly the cell division rates. Other resistant genes to miRNA cleavage, distinct to the *SPL* family, were reported during the establishment of the RMS as positive promoters for cell division; for example, plants with a miR159-resistant form of *MYB65* display higher number of dividing cells in the root meristem by promoting the

transcription of *CYCB1;1* (Xue *et al*., 2017). On the other hand, transgenic plants with the resistant version to the miR396 cleavage for the *GRF2* and *GRF3* genes, display reduction in the RMS by altering not only the *CYCLINB1,* but also by changing the speed of the cell cycle, leading to alteration in the transition of root stem cells into transit-amplifying cells (Rodriguez *et al*., 2015). In this way, our data suggest that *SPL10* participates on the circuit that controls the cell division rates in PR meristem.

5.7 Cytokinin responses are affected by the de-regulation of the *SPL10.*

Cytokinin response was clearly affected in *rSPL10* roots, showed not only by the *ARR1* expression but also by the intensity of cytokinin response signals observed by *TCSn::GFP* and *pARR5:GUS* reporters. Low cytokinin response is in agreement with the enhanced meristem size observed in *rSPL10* roots. Therefore, we suggest that the de-regulation of the *SPL10*-dependent modulation of meristematic cell division may occur through two independent or overlapping mechanisms: (1) by directly increasing the overall division rate (visualized by the higher *pCYCB1;1::GUS* expression levels in *rSPL10* roots); and/or (2) by attenuating the cytokinin responses in roots, thus, decreasing cell differentiation rate. Together, these observations are consistent with the role of cytokinins in controlling the rate of meristematic cell differentiation (Dello Ioio *et al*., 2007, 2008).

Zhang *et al*. (2015) reported that the SPL10 protein directly bind to B-type ARR2 protein, lessening cytokinin responses (Heyl and Schmülling, 2003). This is consistent with our observations that *TCSn::GFP* and *pARR5::GUS* reporter levels were reduced in *rSPL10* roots. The transcriptional activator *B-type ARR1*, along with *ARR12* gene, is crucial for controlling most of the genes under cytokinin regulation (Argyros *et al*., 2008). *ARR1* transcript levels were reduced in *MIM156* and *rSPL10* roots at 10-dpg. This suggests that, in addition to regulating ARR transcriptional activity (Zhang *et al*., 2015), miR156-targeted *SPL*s, including *SPL10*, may modulate cytokinin-responsive genes in roots via *ARR1* transcriptional regulation. However, whether this is achieved by a direct or indirect regulation of *ARR1* deserves further investigation.

6. CONCLUSIONS

The mature miR156 is a master regulator of age-associated plant development throughout plant kingdom which is produced by independent *MIR156* genes. In *Arabidopsis,* the *MIR156* genes express and decrease through time in root tissues as in the aerial part of the plant, by which the mechanisms of the *MIR156* genes regulation may be also conserved and therefore deserve to be explored in more depth.

The reduction of miR156 levels overtime leads to the increase of *SPL* expression in the aerial part and, consequently, contributes to phase transition. In root tissues, the *SPL* expression helps to root growth, and the mechanisms of the *SPL* regulation in roots are also dependent, but probably not exclusive, to age-associated miR156 reduction.

Appropriate miR156 levels promote a suitable primary root growth. The disruption in the mi156/*SPL* pathway leads to phenotypic changes in root system. High levels of the miR156 repress the primary root growth, while low levels promote it; in this manner, it could be a powerful tool for exploring plant

productivity-related researches and directly dependent of primary root growth in agronomic importance species.

The root meristem size-controlled primary root growth is directly associated with the balance between the antagonistic effects of auxin, controlling cell division, and cytokinin, controlling cell differentiation; besides that, miRNAs-controlled genetic pathways also participate in the established of the root meristem size and in the suitable primary root growth.

The miR156 is a small RNA molecule that participates in many different growth and developmental processes throughout the plant kingdom by regulating the expression of the *SPL* genes; in this way, the effects found in the root system of the model plant *Arabidopsis* could be found in other plant species and therefore are worthy of being studied.

According to the available literature, our work constitutes the first study about the interplay between the miR156/*SPL* pathway and the phytohormones auxin and cytokinin during the root meristem size–associated primary root growth in *Arabidopsis*, and contributes to unravel the molecular mechanisms involved in the growth and development of the root system.

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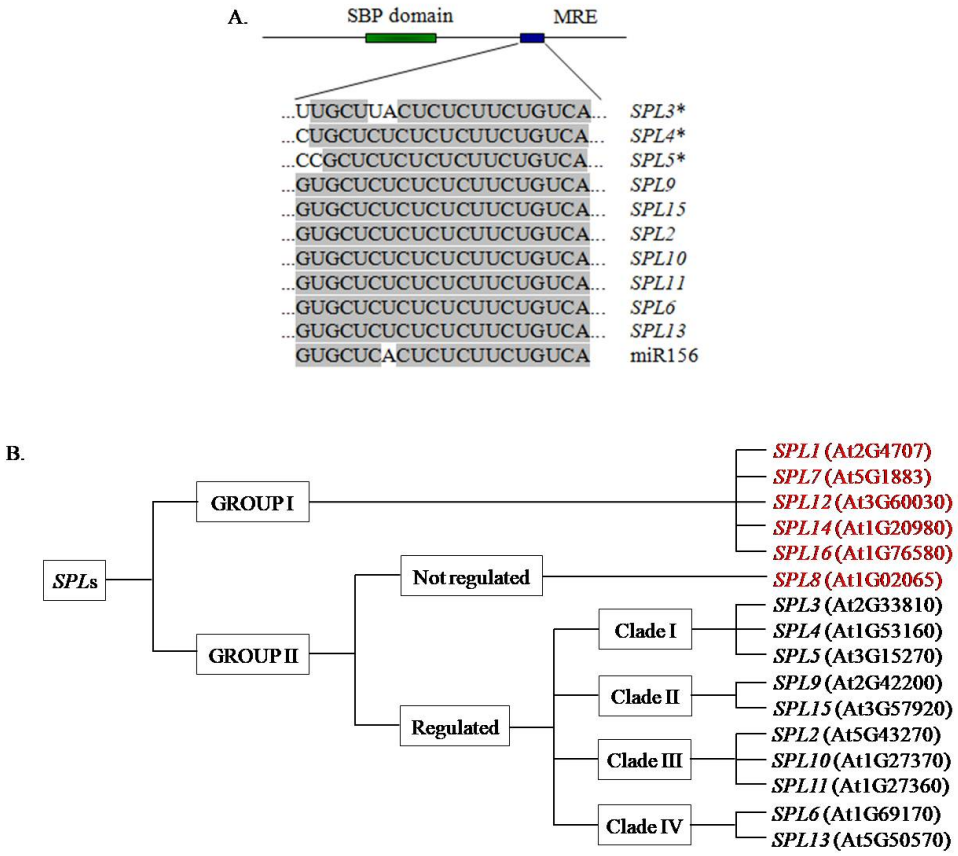
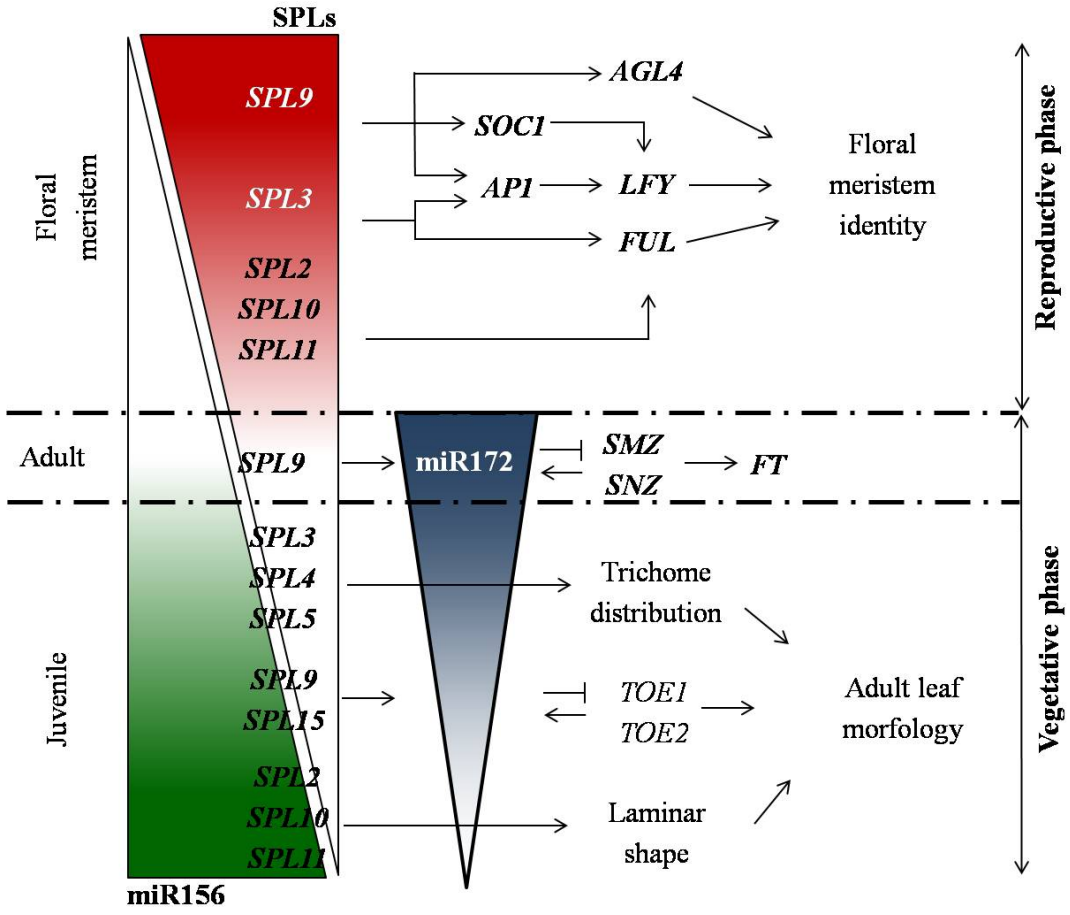
8. APPENDICES



Appendix 1. miR156 responsive element sequence and grouping of the *SPL* family. A.: sequence of the miR156 responsive element (MRE) on the miR156-targeted SPLs genes in *Arabidopsis*. The MRE is located downstream to the *SQUAMOSA* Binding Protein (SBP) domain in most of the *SPL* genes, with exception to the *SPL3**, *SPL4**, and *SPL5**, which is located on the 3'-untranslated region (Cardon *et al*., 1999). B.: grouping of the *SPL* family. In *Arabidopsis*, there are 16 *SPL* genes. 10 out of those *SPL* genes are post-transcriptionally regulated by the miR156 (Rhoades *et al*., 2002). The miR156-targeted *SPL* genes can be grouped into four functional clades, clade I: *SPL3*, *SPL4*, *SPL5*; clade II: *SPL2*, *SPL10*, *SPL11*; clade III: *SPL6*, *SPL13*; and clade IV: *SPL9*, *SPL15* (Guo *et al*., 2008).



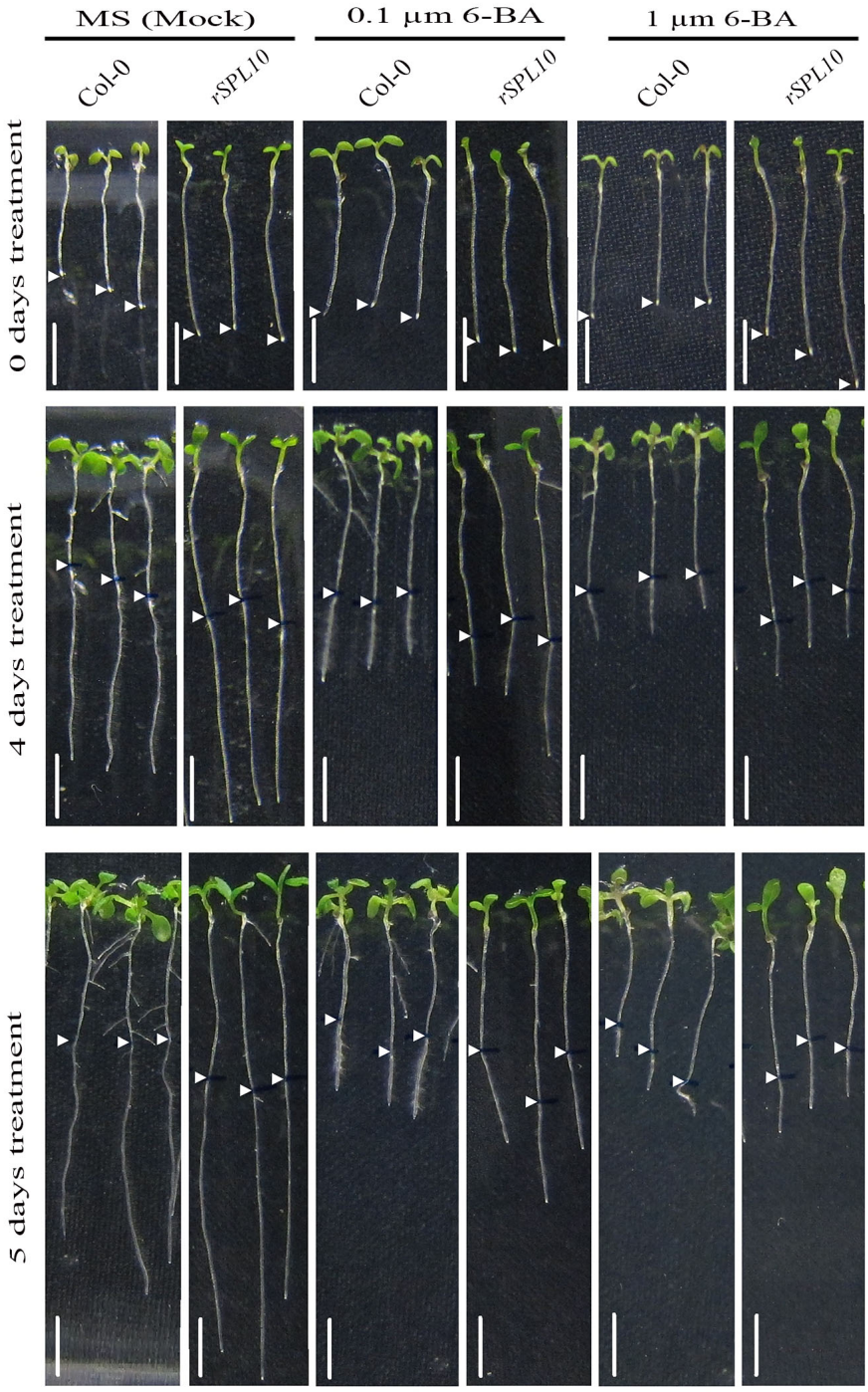
Appendix 2. **miR156 and *SPL* genes controlled-regulation circuit during phase change in *Arabidopsis*.** The plant development is divided in juvenile, adult and reproductive phases. The green gradual shading represents the decreasing of the miR156 leves during time, leading to the increasing of the miR56-regulated *SPL* genes (red gradual shading), and consequently increasing of the SPLs-mediated miR172 leves (blue gradual shading). Lines with arrowhead designate positive regulation, whereas lines with a bar at the end indicate negative regulation (Chen *et al*., 2010)

Appendix 3. Oligonucleotide sequences used in this work

Name	Sequence (5'-3')	Purpose
SPL2_F	CCGACGTCTCTCAGATCACA	RT-PCR
SPL2_R	TGGTACGTGCTTCGAACTTG	RT-PCR
SPL3_F	CTTAGCTGGACACAACGAGAGAAGG	RT-PCR
SPL3_R	GAGAAACAGACAGAGACACAGAGGA	RT-PCR
SPL4_F	CCAAAATGGAGGGTAAGAGA	RT-PCR
SPL4_R	GCCTCTTTCATATCAGCTGTGC	RT-PCR
SPL5_F	ATGCAGCAGGTTTCATGAGC	RT-PCR
SPL5_R	GCCTGACCCTTCTCCAAAAC	RT-PCR
SPL6_F	CTTGGAGCTACGGGAGAAGC	RT-PCR
SPL6_R	TCCATTGGAGTTACCAGCCA	RT-PCR
SPL9_F	GGTCGGGTCAGTCGGGTCAGATACC	RT-PCR - qRT-PCR
SPL9_R	ACTGGCCGCCTCATCACTCTTGTATCC	RT-PCR - qRT-PCR
SPL10_F	GTGGGAGAATGCTCAGGAGGC	RT-PCR - qRT-PCR
SPL10_R	GAGTGTGTTTGATCCCTTGTGAATCC	RT-PCR - qRT-PCR
SPL11_F	GTCCAAGTTTCAACTTCATGGCG	RT-PCR

SPL11_R	GAACAGAGTAGAGAAAATGGCTGCAC	RT-PCR
SPL13_F	CCAATCTCTTCTTCTCCAAACAGTACC	RT-PCR
SPL13_R	GAAGCAAATGAGGGACTGACGACG	RT-PCR
SPL15_F	TGAATGTTTTATCACATGGAAGCTC	RT-PCR
SPL15_R	TCATCGAGTCGAAACCAGAAGAT	RT-PCR
MIR156A-F	CTTCGTTCTCTATGTCTCAATCTCTC	qRT-PCR
MIR156A-R	TGATTAAAGGCTAAAGGTCTCCTC	qRT-PCR
MIR156C-F	GTGATAATGAGTGATGACTGATG	qRT-PCR
MIR156C-R	GAAAACGTGACCGGGACCGAATCG	qRT-PCR
miR156_F	CCTGAGTGACAGAAGAGAGTG	RT-PCR - qRT-PCR
Rev. Universal	GTGCAGGGTCCGAGG	RT-PCR - qRT-PCR
Stem-loop miR156	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCTCT	cDNA synthesis
PIN1_F	CGTGGAGAGGGAAGAGTTTA	qRT-PCR
PIN1_R	AACATAGCCATGCCTAGACC	qRT-PCR
PIN3_F	TCTTTGATTAGGTTCGGGTAACTC	qRT-PCR
PIN3_R	GCTCATGTGAAACTGGAACAAG	qRT-PCR
PIN7_F	CCAAGATTAGTGGAACGCAAC	qRT-PCR

PIN7_R	GAAAAGGGTTTTTGGATCCTC	qRT-PCR
ARR1_F	GGAAACGGTGGTTCAGTGAGGGT	qRT-PCR
ARR1_R	CGATGGAGTATGCGTCAAAGTCG	qRT-PCR
ARR12_F	CTTCAAGGGTTGCCAATGCC	qRT-PCR
ARR12_R	GAAGCCTGGAGGAGTTGCTT	qRT-PCR
CYCB1_F	CGATCTCAAAATCCCACGCTTC	qRT-PCR
CYCB1_R	GGTCGCTTTCTTCTTAGTAGCC	qRT-PCR
RCH1_F	ATTAGGCGTGTTGGCGGTTA	qRT-PCR
RCH1_R	GAAAAGGTGTGAATTGCCATGTC	qRT-PCR
ACTIN2-F	GACCTTGCTGGACGTGACCTTAC	RT-PCR - qRT-PCR
ACTIN2-R	GTAGTCAACAGCAACAAAGGAGAGC	RT-PCR - qRT-PCR



Appendix 4. Representative pictures of Col-0 and *rSPL10* seedlings in response to 6-Benzyladenine (6-BA) treatments. Seedlings at 5-dpg (0 days of treatment) were transferred to MS medium supplemented with 0.1 µm or 1.0 µm of 6-BA. Roots were photographed at 4- and 5-days post-treatment (dpt) and the increments in root length were measured from the initial growth region (arrowheads) using the ImageJ software; scale bar: 1cm.