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BOTUCATU-SP

2019

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Thesis submitted to the Institute of Biosciences, Botucatu Campus, UNESP, for obtaining the doctoral degree in Biological Sciences (Genetics).

BOTUCATU – SP

2019

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP

BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Barrera Rojas, Carlos Hernán.
The role of the microRNA156/*SPL* pathway during the primary root growth of *Arabidopsis thaliana* / Carlos Hernán Barrera Rojas. - Botucatu, 2019

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Fábio Tebaldi Silveira Nogueira
Capes: 20200005

1. *Arabidopsis*. 2. Reguladores de crescimento de planta. 3. MicroRNAs. 4. Raízes (Botânica). 5. Plantas e solo.

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

**CONTENTS**

## ACKNOWLEDGEMENTS

I would like to thank all the people and institutions that contributed to the development of this study, especially to:

- God, my parents and my family, for being the reason of my life, the force that leads me to continue.
- My advisor Fabio TS Nogueira for accepting and believing in me, and for introducing me to the world of the molecular genetics of plant development.
- The graduate program in Biological Sciences (Genetics) at the Institute of Biosciences (Botucatu Campus), *Universidade Estadual Paulista "Júlio de Mesquita Filho"* for accepting me as a graduate student.
- The National Council for Scientific and Technological Development (*CNPq*) from Brazil for granting me the scholarship.
- The research group on Molecular Genetics of Plant Development (*GMDV*), for the teachings, advices, cooperation, and patience.

- The staff of the Graduate Program in Biological Sciences (Genetics) from the Institute of Biosciences (Botucatu) for the administrative support.

- The "*Luiz de Queiroz*" College of Agriculture (ESALQ) from University of São Paulo for allowing me to conduct my experiments on this campus.

- The Microscopy Unit from ESALQ for allowing me to obtain the microphotographs for this study.

- Sabrina Sabatini from the University of Rome (*La Sapienza*) for helping us to conduct the root meristem analyzes.

- The members of the evaluation committee for evaluating this study.

- My brazilian friends for the patience and teachings in my portuguese.

- All my friends for being part of this adventure.

- You, for reading this study.

**THANK YOU!**

**Dedicated to my parents Antonio and Rosa**

## 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 |

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## 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).

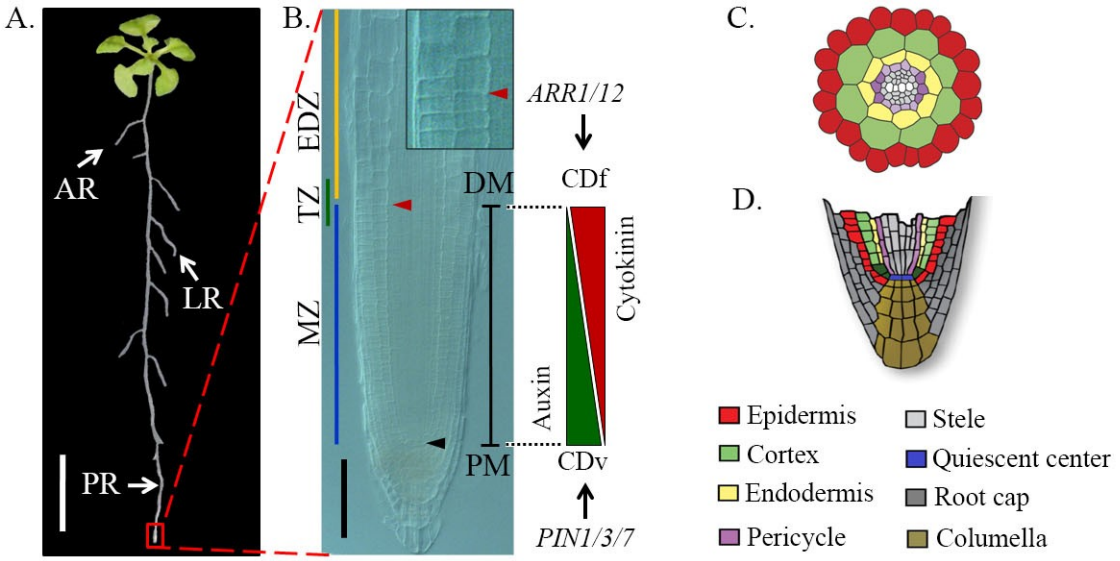


**Figure 1. The root system in *Arabidopsis thaliana.*** A.: representative picture of the root system in Col-0 at 12-days-post-germination (dpg), indicating the adventitious root (AR), lateral root (LR), and the primary root (PR); scale bar: 1 cm. B.: Light microscope picture of the longitudinal view 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).

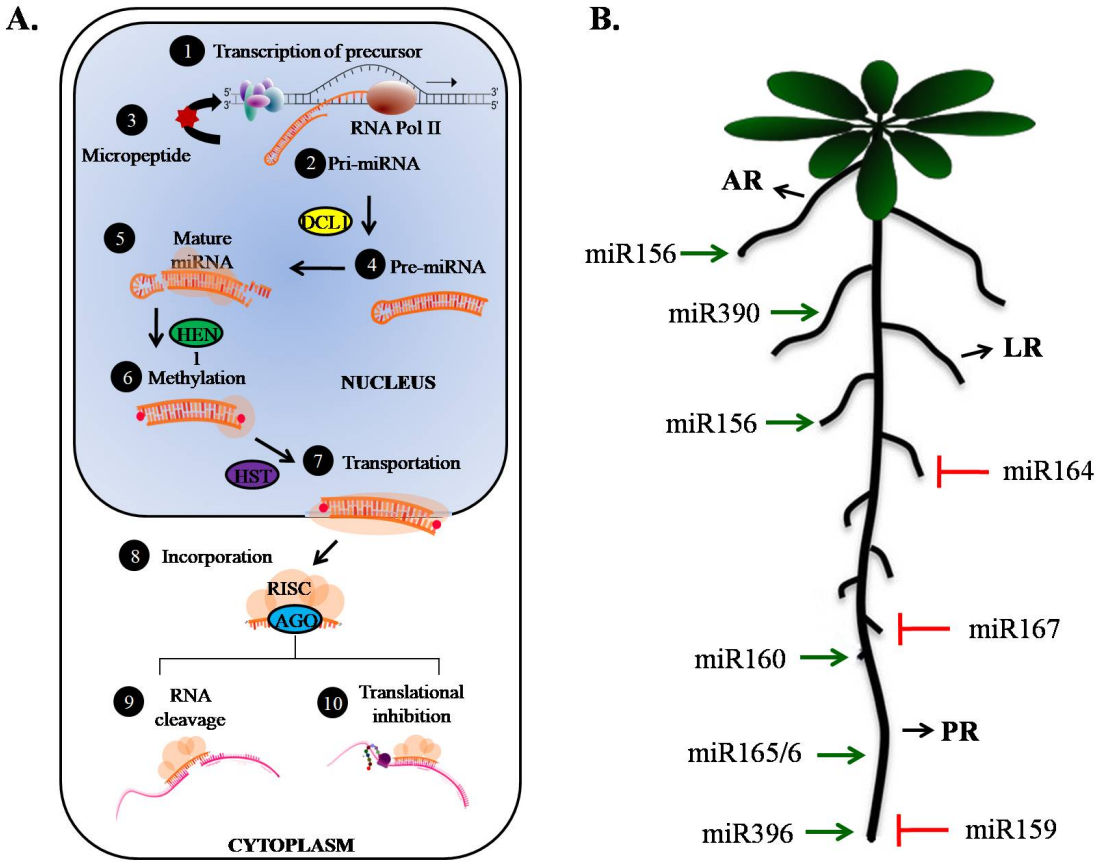


**Figure 2. Biogenesis and miRNAs in *Arabidopsis* root growth and development.** A.: the biogenesis of the miRNAs in plants. After the RNA polymerase II-mediated transcription of precursor (1), it is generated a single stranded precursor named pri-miRNA (2). In some cases, this precursor encodes a micro-peptide 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*.

# 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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