KERLY JESSENIA MONCALEANO ROBLEDO

ROLE OF THE miR156/SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) PATHWAY IN GROWTH AND DEVELOPMENT Of *Passiflora cincinnata* MAST. (PASSIFLORACEAE)

Thesis submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Wagner Campos Otoni

VIÇOSA - MINAS GERAIS
2022

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T

M737r
2022

Moncaleano Robledo, Kerly Jessenia, 1991-

Role of the miR156/Squamosa Promoter-Binding Protein-Like (SPL) pathway in growth and development of *Passiflora cincinnata* Mast. (Passifloraceae) / Kerly Jessenia Moncaleano Robledo. – Viçosa, MG, 2022.

1 tese eletrônica (66 f.): il. (algumas color.).

Texto em inglês.
Orientador: Wagner Campos Otoni.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Biologia Vegetal, 2022.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2023.098
Modo de acesso: World Wide Web.

1. Botânica - Morfologia. 2. Passiflora - Desenvolvimento. 3. Marcadores genéticos. 4. Gavinhas. 5. Nectários. I. Otoni, Wagner Campos, 1962-. II. Universidade Federal de Viçosa. Departamento de Biologia Vegetal. Programa de Pós-Graduação em Fisiologia Vegetal. III. Título.

CDD 22. ed. 571.3

Bibliotecário(a) responsável: Euzébio Luiz Pinto CRB-6/3317

KERLY JESSENIA MONCALEANO ROBLEDO

ROLE OF THE miR156/SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) PATHWAY IN GROWTH AND DEVELOPMENT Of *Passiflora cincinnata* MAST. (PASSIFLORACEAE)

Thesis submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

APPROVED: November 28th, 2022.

Assent:

gov.br
Documento assinado digitalmente
KERLY JESSENIA MONCALEANO ROBLEDO
Data: 26/03/2023 19:19:00-0300
Verifique em https://validar.iti.gov.br

Kerly Jessenia Moncaleano Robledo
Author

gov.br
Documento assinado digitalmente
WAGNER CAMPOS OTONI
Data: 26/03/2023 18:27:22-0300
Verifique em https://validar.iti.gov.br

Wagner Campos Otoni
Adviser

ACKNOWLEDGMENTS

I thank my parents, Martha and Carlos, and my sisters, Claudia, Yasmin, and Cristina, for their love, the example of strength, work, and dedication.

To my husband Camilo Guzman and my daughter Sophia Guzman for the support, love, and patience, especially on difficult days.

To the Universidade Federal de Viçosa (UFV) for the infrastructure for developing this research and to the Plant Physiology Program, and to all the teachers that contributed to my academic formation.

To Prof. Wagner Otoni for the guidance and teachings during project development. I will always be grateful for the comprehension, affection, respect, and friendship we shared.

To Prof. Fabio Nogueira for the scientific inputs and advice on this project.

To the Laboratory of Plant Anatomy of the UFV for providing the material and equipment for the performance of anatomical analyzes and histochemical tests.

To the laboratory of Ecology and prof. Carlos Sperber for allowing me to use the stereomicroscope.

To Professor Lyderson Facio Viccini for providing the Genetics and Biotechnology Laboratory (LGB) of the Federal University of Juiz de Fora to carry out the flow cytometry analysis.

To my friends Jessica Soares, Tatiane da Silva, Daniele Vidal, and Evandro Fortini for the discussions and help in sampling and analysis.

To my friends Emmanuel Naves, Juliene Moreira, Suzy Barbosa, Bruno Rosa, Hellen Oliveira, and the entire LCT II team for the company, friendship, and advice.

To my colleagues from the Plant Physiology graduate program for the conversations, help, and companionship.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001, who also granted my scholarship, and byFundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) –grants no. APQ-02372-17 and APQ-00772-19.

ABSTRACT

ROBLEDO, Kerly Jessenia Moncaleano, D.Sc., Universidade Federal de Viçosa, November, 2022**.** **Role of the miR156/SQUAMOSA promoter-binding protein-like (SPL) pathway in growth and development of *Passiflora cincinnata* Mast. (Passifloraceae)**. Adviser: Wagner Campos Otoni.

Post-embryonic plant development can be divided into vegetative, juvenile, adult, and reproductive phases. The transition of these phases is modulated by exogenous and endogenous factors, with the microRNA156 (miR156) pathway playing a central role in maintaining the juvenile phase by regulating genes of the SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SBP/SPL) family of transcription factors. In the process of phase transition, multiple phenotypic changes occur, and in Passiflora cincinnata, besides leaf changes, the appearance of tendrils stands out. These structures are of great physiological and ecological relevance by favoring the performance and mutualistic interactions, e.g., with herbivores or pollinators and competition for resources with other species. However, the genetic and molecular control of their development is still unknown, as well as the possible role of miR156 in the growth and development of such structures. Thus, *P. cincinnata* is an excellent model for studying the molecular basis of nectaries and tendril development. It is necessary to generate overexpression (*OE::miR156*) and silencing (*STTM156*) lines that facilitate understanding the effects of the miR156/*SPLs* module. Our results show that in *P. cincinnata*, inhibition in phase transition is reflected in the maintenance of the juvenile stage in *OE::miR156* plants compared to untransformed lines (NT). The miR156/SPL module affected the growth of tendrils. In *OE::miR156* plants, initial development is maintained at the same growth rate as NT, but development is inhibited upon reaching approximately one-third of growth. In contrast, there is a continuity of growth in NT and silenced lines. Likewise, the emission of the first tendril did not show dependence on miR156 levels and represented the adult stage as believed, being more linked to the number of developed phytomers. Being a climbing specie is essential to emit this organ early in development to find support and allow resource exploitation. On the other hand, in the *OE::miR156* plants, the leaves were permanently juvenile, which for *P. cincinnata* corresponds to trilobed leaves, contrasting with NT and the silenced lines that when transitioning phase issued pentalobed leaves. In later phytomeres, pentalobate leaves show greater complexity in NT plants, and this is accompanied by a greater number of nectaries, in contrast to the silenced lines. Demonstrating that leaf and nectary development are closely related. Thus, our observations show that miR156 affects the growth and development of nectaries and tendrils *in P. cincinnata*, and there is fine regulation by the aging pathway.

Keywords: *Passiflora.* miRNA. Tendrils. Extrafloral nectaries. Development.

RESUMO

MONCALEANO ROBLEDO, Kerly Jessenia, D.Sc., Universidade Federal de Viçosa, novembro de 2022. **Papel da via miR156/SQUAMOSA PROMOTER BINDING (SPL) no crescimento e desenvolvimento de *Passiflora cincinnata* Mast. (Passifloraceae).** Orientador: Wagner Campos Otoni.

O desenvolvimento vegetal pós-embrionário pode ser dividido em fases vegetativa, juvenil, adulta e reprodutiva. A transição destas fases é modulada por fatores exógenos e endógenos, sendo que a via do microRNA156 (miR156) tem papel central na manutenção da fase juvenil regulando genes da família de fatores de transcrição *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SBP/SPL*). No processo de transição de fases, múltiplas mudanças fenotípicas ocorrem e em *Passiflora cincinnata* se destaca além de mudanças foliares o aparecimento de gavinhas. Ditas estruturas são de grande relevância fisiológica e ecológica ao favorecer o desempenho e as interações de mutualismo, p.ex com herbívoros ou polinizadores e de competição por recursos com outras espécies. Porém, o controle genético e molecular do seu desenvolvimento ainda é desconhecido, assim como o possível papel do miR156 no crescimento e desenvolvimento de ditas estruturas. Assim, *P. cincinnata* é um excelente modelo para estudar as bases moleculares de desenvolvimento de nectários e gavinhas, sendo necessário gerar linhagens de super-expressão (*OE::miR156*) e silenciamento (*STTM156*) que facilitem a compreensão dos efeitos do módulo miR156/SPLs. Nossos resultados mostram que em *P. cincinnata* a inibição na transição de fases se reflete na manutenção do estágio juvenil nas plantas *OE::miR156* em comparação com linhas não transformadas (NT). O módulo miR156/*SPL* afetou o crescimento das gavinhas, sendo que em plantas *OE::miR156* o desenvolvimento inicial é mantido na mesma taxa de crescimento que o NT, porém, ao atingir aproximadamente um terço do crescimento o desenvolvimento é inibido, enquanto em NT e linhas silenciadas há continuidade do crescimento. Assim mesmo, a emissão da primeira gavinha não demonstrou dependência dos níveis de miR156 e representar a fase adulta como se acreditava, estando mais ligado ao número de fitômeros desenvolvidos. Ao ser uma espécie escaladora, é essencial emitir esse órgão no início do desenvolvimento para encontrar suporte e permita a exploração de recursos. Por outro lado, nas plantas *OE::miR156* as folhas foram permanentemente juvenis, que para *P. cincinnata* corresponde a folhas trilobadas, contrastando com NT e as linhas silenciadas que ao transicionar de fase emitiram folhas pentalobadas. Nos fitômeros posteriores, as folhas pentalobadas apresentam maior complexidade em plantas NT e isto é acompanhado por um maior número de nectários, em contraste com as linhas silenciadas. Demonstrando que o desenvolvimento foliar e de nectários estão intimamente relacionados. Desta forma, as nossas observações mostram que miR156 afeta o crescimento e desenvolvimento de nectários e gavinhas em P. cincinnata, existindo uma fina regulação pela rota da idade.

Palavras-chave: *Passiflora*. miRNA. Gavinha. Nectários extraflorais, Desenvolvimento.

CONTENTS

GENERAL INTRODUCTION

Plants undergo several developmental transitions during their life cycle. The juvenile vegetative phase begins with germination, representing the transition from embryonic to post-embryonic growth mode. After germination, the seedling goes through a fast-growing juvenile vegetative phase, where it is not yet competent to flower. This stage is succeeded by the adult vegetative phase, at which point it can respond to inductive floral signals. Subsequently, there is a transition to flowering in which the reproductive stage is reached (Huijser and Schmid, 2011).

The progress through these successive phases involves gradual changes in shoot development, which may present different morphological characteristics throughout the transition (Poethig, 2013). Thus, the transition is modulated by endogenous factors such as carbohydrates and phytohormones associated with microRNAs (miRNAs) (Liu and Chen, 2009; Yu et al., 2013; Poethig, 2013). Among the exogenous factors, luminosity, temperature, and photoperiod stand out, influencing the transition time to align development with favorable conditions and maximizing reproductive success (Bäurle and Dean, 2006).

In some species, there are substantial changes in morphology between the juvenile and adult phases in the vegetative period, reflecting the temporal development of the shoot apical meristem from which the leaves are derived, a phenomenon called heteroblasty (Wang et al., 2011; Cutri et al., 2013; Poethig, 2013; Silva et al., 2019b). These changes are particularly evident in some woody species that are favorable for the study of vegetative phase change due to the stability and prolonged duration of several stages in shoot development that facilitates the observation and characterization of these phases (Scott Poethig, 2010); in contrast, the model species Arabidopsis, in a few days, shows multiple modifications. However, in both cases, time can be a disadvantage in analyzing the physiological, anatomical, and molecular responses throughout development.

The miRNAs are one of the pathways that allow the study of phase transition and organ development associated with age in plants. miRNAs are widely distributed in terrestrial plants and have recently been recognized to play a significant role in gene expression regulation by inhibiting the translation of target mRNAs into proteins or targeting them for degradation (Grotewold et al., 2015). Hundreds of targets genes regulated by miRNAs, in turn, participate in the regulation of developmental genes such as stem and root apical meristem regulation (Williams, 2005; Eun et al., 2011; Fouracre and Poethig, 2019), embryo development zygotic (Gross et al., 2017), somatic embryogenesis (Siddiqui et al., 2019), organogenesis (Liu et al., 2014), phase transition (Wu and Poethig, 2006; Wu et al., 2009; Wang et al., 2011; Fouracre and Poethig, 2016), floral development (Cartolano et al., 2007), tissue differentiation (Silva et al., 2014), regulation of the adaxial/abaxial leaf pattern (Fouracre and Poethig, 2016), shoot regenerative capacity (Zhang et al., 2015) and, in some cases, stress tolerance (Cui et al., 2015; Li et al., 2016; Ni et al., 2012).

Two key miRNAs, miR156 and miR172, act as significant orchestrators in the aging pathway. miR156 plays a crucial role in a modulating phase transition, being highly conserved in angiosperms (Ortiz Morea, 2013). Its targets are the family of transcription factors *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE - SBP/SPL* (Xing et al., 2010). SPLs genes play a multifunctional role in plant development and growth and are involved in the transition from juvenile to vegetative adult stages (Wu and Poethig, 2006), promoting the transcription of miR172 and genes such as *FRUIT-FULL (FUL), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), LEAFY (LFY), AGAMOUS-LIKE (AGL24) and APETALA 1 (AP1)*. In turn, miR172 represses the expression of repressor genes *APETAL*A 2 TARGET OF EAT 1/2, SCHLAFMÜTZE (SMZ), and SCHNARCHZAPFEN (SNZ), which repress the floral inducer *Flowering Locus T (FT*) (Chen, 2004; Schwab et al., 2005; Zhu and Helliwell, 2011; Wu et al., 2009).

During growth and development, hormones play an important signaling role and mediate response to biotic and abiotic factors. In some processes mentioned above, several miRNAs are crucial regulators of the commonly studied phytohormones that have been described, regulating the level of biosynthesis, transport, and perception. (Liu and Chen, 2009; Curaba et al., 2014).

Advances in research into the role of miR156 in several plant species through overexpression and silencing strategies have shown that, in general, high levels of miR156 lead plants to show a determined growth habit, maintenance of juvenile characteristics, reduction of inhibition correlative with a consequent increase in lateral branching and delay in flowering, among other altered physiological characteristics (Xie et al., 2012; Wu and Poethig, 2006; Feng et al., 2016; Aung et al., 2015). In the case of *Solanum lycopersicum*, in addition to the previously described effects on growth, it was shown that the morphology of flowers and fruits was abnormal, characterized by the growth of extra carpels and ectopic structures (Silva et al., 2014).

Another approach that increases the knowledge of an organism's physiological, morphological, and molecular processes is sequencing its transcripts. The exploration of the transcriptome from next-generation sequencing technologies provides a remarkable resource for identifying genes differentially expressed in response to treatment and, subsequently, the elucidation of biosynthetic pathways and is especially interesting in non-model species, which do not count on known genomic sequences like *Passiflora edulis* (Liu et al., 2017), *Bixa orellana* (Cárdenas-Conejo et al., 2015), *Pfaffia glomerata* (Batista et al., 2019) and *Arceuthobium sichuanense* (Wang et al., 2016).

However, the effect of the miR156/SPL module on global gene expression, as well as the morphological and anatomical differences that occur in *P. cincinnata* under conditions of repressed SPLs (miR156 superexpression) and free SPLs (miR156 sequestration), are unknown. Thus, the assays performed in this thesis demonstrate the hypothesis that miR156 impacts the growth and development of *P. cincinnata* at the organelle and whole-plant level and may affect interspecific relations.

Accordingly, this thesis is divided into two chapters. Here, we show the effects of the miR156/SPLs module on the growth and development of *P. cincinnata*, focusing on tendrils and nectaries.

REFERENCES

Aung, B., Gruber, M.Y., Amyot, L., Omari, K., Bertrand, A., and Hannoufa, A. (2015). Ectopic expression of LjmiR156 delays flowering, enhances shoot branching, and improves forage quality in alfalfa. Plant Biotechnol. Rep. **9**: 379–393.

Batista, D.S. et al. (2019). De novo assembly and transcriptome of *Pfaffia glomerata* uncovers the role of photoautotrophy and the P450 family genes in 20-hydroxyecdysone production. Protoplasma **256**: 601–614.

Bäurle, I. and Dean, C. (2006). The timing of developmental transitions in plants. Cell **125**: 655–664.

Cárdenas-Conejo, Y., Carballo-Uicab, V., Lieberman, M., Aguilar-Espinosa, M., Comai, L., and Rivera-Madrid, R. (2015). De novo transcriptome sequencing in *Bixa orellana* to identify genes involved in methylerythritol phosphate, carotenoid and bixin biosynthesis. BMC Genomics **16**: 1–19.

Cartolano, M., Castillo, R., Efremova, N., Kuckenberg, M., Zethof, J., Gerats, T., Schwarz-Sommer, Z., and Vandenbussche, M. (2007). A conserved microRNA module exerts homeotic control over *Petunia hybrida* and Antirrhinum majus floral organ identity. Nat. Genet. **39**: 901–905.

Chen, X. (2004). A MicroRNA as a translational repressor of *APETALA2* in Arabidopsis flower development. Science (80-.). **303**: 2022–2025.

Cui, N., Sun, X., Sun, M., Jia, B., Duanmu, H., Lv, D., Duan, X., and Zhu, Y. (2015). Overexpression of OsmiR156k leads to reduced tolerance to cold stress in rice (*Oryza Sativa*). Mol. Breed. **35**: 1–11.

Curaba, J., Singh, M.B., and Bhalla, P.L. (2014). MiRNAs in the crosstalk between phytohormone signalling pathways. J. Exp. Bot. **65**: 1425–1438.

Cutri, L., Nave, N., Ami, M. Ben, Chayut, N., Samach, A., and Dornelas, M.C. (2013). Evolutionary, genetic, environmental and hormonal-induced plasticity in the fate of organs arising from axillary meristems in Passiflora spp. Mech. Dev. **130**: 61–69.

Eun, C., Lorkovic, Z.J., Naumann, U., Long, Q., Havecker, E.R., Simon, S.A., Meyers, B.C., Matzke, A.J.M., and Matzke, M. (2011). AGO6 functions in RNA-mediated transcriptional gene silencing in shoot and root meristems in *Arabidopsis thaliana*. PLoS One **6**: e25730.

Feng, S., Xu, Y., Guo, C., Zheng, J., Zhou, B., Zhang, Y., Ding, Y., Zhang, L., Zhu, Z., Wang, H., and Wu, G. (2016). Modulation of miR156 to identify traits associated with vegetative phase change in tobacco (*Nicotiana tabacum*). J. Exp. Bot. **67**: 1493–1504.

Fouracre, J.P. and Poethig, R.S. (2016). The role of small RNAs in vegetative shoot development. Curr. Opin. Plant Biol. **29**: 64–72.

Fouracre, J.P. and Scott Poethig, R. (2019). Role for the shoot apical meristem in the specification of juvenile leaf identity in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. **116**: 10168–10177.

Gross, N., Kropp, J., and Khatib, H. (2017). MicroRNA signaling in embryo development. Biology (Basel). **6**.

Grotewold, E., Chappell, J., and Kellogg, E.A. (2015). Plant genes, genomes and genetics 1st ed. (Wiley).

Huijser, P. and Schmid, M. (2011). The control of developmental phase transitions in plants. Development **138**: 4117–4129.

Li, W., Wang, T., Zhang, Y., and Li, Y. (2016). Overexpression of soybean miR172c confers tolerance to water deficit and salt stress, but increases ABA sensitivity in transgenic *Arabidopsis thaliana*. J. Exp. Bot. **67**: 175–194.

Liu, Q. and Chen, Y.Q. (2009). Insights into the mechanism of plant development: Interactions of miRNAs pathway with phytohormone response. Biochem. Biophys. Res. Commun. **384**: 1–5.

Liu, S., Li, A., Chen, C., Cai, G., Zhang, L., Guo, C., and Xu, M. (2017). De novo transcriptome sequencing in *Passiflora edulis* sims to identify genes and signaling pathways involved in cold tolerance. Forests **8**: 435.

Liu, W., Yu, W., Hou, L., Wang, X., Zheng, F., Wang, W., Liang, D., Yang, H., Jin, Y., and

Xie, X. (2014). Analysis of miRNAs and their targets during adventitious shoot organogenesis of *Acacia crassicarpa*. PLoS One **9**: e93438.

Ni, Z., Hu, Z., Jiang, Q., and Zhang, H. (2012). Overexpression of gma-MIR394a confers tolerance to drought in transgenic *Arabidopsis thaliana*. Biochem. Biophys. Res. Commun. **427**: 330–335.

Ortiz Morea, E.G. (2013). Papel funcional de microRNAs na arquitetura vegetativa e radicular de plantas. Proc. Natl. Acad. Sci. U. S. A.

Poethig, R.S. (2013). Vegetative phase change and shoot maturation in plants. In Current Topics in Developmental Biology (Academic Press Inc.), pp. 125–152.

Schwab, R., Palatnik, J.F., Riester, M., Schommer, C., Schmid, M., and Weigel, D. (2005). Specific effects of microRNAs on the plant transcriptome. Dev. Cell **8**: 517–527.

Scott Poethig, R. (2010). The past, present, and future of vegetative phase change. Plant Physiol. **154**: 541–544.

Siddiqui, Z.H., Abbas, Z.K., Ansari, M.W., and Khan, M.N. (2019). The role of miRNA in somatic embryogenesis. Genomics **111**: 1026–1033.

Silva, G.F.F.E., Silva, E.M., Da Silva Azevedo, M., Guivin, M.A.C., Ramiro, D.A., Figueiredo, C.R., Carrer, H., Peres, L.E.P., and Nogueira, F.T.S. (2014). MicroRNA156-targeted SPL/SBP box transcription factors regulate tomato ovary and fruit development. Plant J. **78**: 604–618.

Silva, P.O., Batista, Di.S., Cavalcanti, J.H.F., Koehler, A.D., Vieira, L.M., Fernandes, A.M., Barrera-Rojas, C.H., Ribeiro, Di.M., Nogueira, F.T.S., and Otoni, W.C. (2019). Leaf heteroblasty in *Passiflora edulis* as revealed by metabolic profiling and expression analyses of the microRNAs miR156 and miR172. Ann. Bot. **123**: 1191–1203.

Wang, J.W., Park, M.Y., Wang, L.J., Koo, Y., Chen, X.Y., Weigel, D., and Poethig, R.S. (2011). MiRNA control of vegetative phase change in trees. PLoS Genet. **7**: e1002012.

Wang, Y., Li, X., Zhou, W., Li, T., and Tian, C. (2016). De novo assembly and transcriptome characterization of spruce dwarf mistletoe *Arceuthobium sichuanense* uncovers gene expression profiling associated with plant development. BMC Genomics **17**: 1–14.

Williams, L., Grigg, S.P., Xie, M., Christensen, S., and Fletcher, J.C. (2005). Regulation of Arabidopsis shoot apical meristem and lateral organ formation by microRNA miR166g and its *AtHD-ZIP* target genes. Development **132**: 3657–3668.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.W., Weigel, D., and Poethig, R.S. (2009). The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell **138**: 750–759.

Wu, G. and Poethig, R.S. (2006). Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. Development **133**: 3539–3547.

Xie, K., Shen, J., Hou, X., Yao, J., Li, X., Xiao, J., and Xiong, L. (2012). Gradual increase of miR156 regulates temporal expression changes of numerous genes during leaf development in rice. Plant Physiol. **158**: 1382–1394.

Xing, S., Salinas, M., Höhmann, S., Berndtgen, R., and Huijser, P. (2010). miR156-targeted and nontargeted SBP-Box transcription factors act in concert to secure male fertility in Arabidopsis. Plant Cell **22**: 3935–3950.

Yu, S., Li, C., Zhou, C.M., Zhang, T.Q., Lian, H., Sun, Y., Wu, J., Huang, J., Wang, G., and Wang, J.W. (2013). Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. Elife **2013**: 1–17.

Zhang, T.Q., Lian, H., Tang, H., Dolezal, K., Zhou, C.M., Yu, S., Chen, J.H., Chen, Q., Liu, H., Ljung, K., and Wang, J.W. (2015). An intrinsic microRNA timer regulates progressive decline in shoot regenerative capacity in plants. Plant Cell **27**: 349–360.

Zhu, Q.H. and Helliwell, C.A. (2011). Regulation of flowering time and floral patterning by miR172. J. Exp. Bot. **62**: 487–495.

CHAPTER I

Effects of miR156/SPLs pathway in growth and development of plants of *Passiflora cincinnata* Mast. (Passifloraceae) and its impact on tendrils

ABSTRACT

The miR156-SPL-mediated plant age pathway is involved in many biological processes regulating its downstream targets, the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SBP/SPL)* family at transcript level or through protein interaction. These processes include organogenesis, root forming, adventitious shoot, response to pathogens, phase transition, flowering time, and response to abiotic stress, among others. Leaf shape among *Passiflora* species is widely diverse, and during the phase transition, some species of the genus show evident morphological differences in the patterned leaf. *P. cincinnata* is characterized by trilobed leaves in the juvenile phase and pentalobed from the adult vegetative phase. In addition to changes in leaf format, tendrils (organs shared among the Passifloraceae family) are recognized as transition markers from the juvenile to the adult phase. These organs also promote plant support and facilitate climbing to the top of the forest, which is an adaptative advantage to improving plant fitness. *Passiflora cincinnata* is a species of climbing perennial found in the interior or edges of dense forests and shrublands, where vertical growth is essential to reach the top and compete for light. Here, our objective was to identify differences in tendril growth and development mediated by age through the miR156/SPLs module. Our results show that repressing SPLs triggered a regulatory network in different organs that caused changes in the phase transition, reflected in changes in the leaf pattern, plant architecture, and in tendril growth and development. miR156/SPLs module is not central in integrating age signals to promote tendril appearance but affects their development and, therefore, the successful support and climbing.

Keywords: Passiflora, miRNA, *SPLs*, tendril, development.

INTRODUCTION

The microRNAs are small regulatory RNA molecules that control gene expression of their target genes through interactions between miRNA-mRNA (Voinnet, 2009) and are widely distributed in plants and animals miRNAs are conservated in plants. Among species of horticultural importance (Chen et al., 2018), and participate in different biological processes in plants like the phase transition, root forming, adventitious shoot organogenesis, and flower development (Liu et al., 2014; Silva et al., 2019a; Wu and Poethig, 2006). Age pathway is mediated by miRNA156, and its targets, where the miR156 negatively regulates the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SBP/SPL)* family of transcription factors that regulate the transition juvenile - adult vegetative and later, as miR156 levels decline, SPLs and miR172 levels increase, leading to the vegetative-reproductive adult phase transition (Wu and Poethig, 2006). Besides the role in the transition, there are also reports on the regulation of other development processes such as leaf architecture (Rubio-Somoza et al., 2014), flowering time (Wu and Poethig, 2006), immunity (Kus et al., 2002), response to pathogens, and tolerance to abiotic stress (Khraiwesh et al., 2012).

Some species of the genus *Passiflora* show evident morphological differences in the leaves during the phase transition, reflecting the temporal development of the apical meristem, a phenomenon called heteroblasty (Chitwood and Otoni, 2017). *Passiflora edulis* have been described, in addition to changes in leaf format, the tendrils, as transition markers from the juvenile to the adult phase (Scorza et al., 2017; Cutri et al., 2013; Nave et al., 2010). Tendrils are organs shared among the Passifloraceae family, which are formed in the axillary meristem and are straightforward structures with a threadlike shape and can coil into other structures through helical growth, promoting plant support and facilitating climbing to the top of the forest (Darwin, 1875). Moreover, some climbing plant species can escape herbivore mammals commonly found on the ground surface, avoiding damage (González-Teuber and Gianoli, 2008; Gianoli and Molina-Montenegro, 2005). Thus, tendrils can be considered an adaptive advantage to improving the climbing fitness of plants.

P. cincinnata is a species of climbing perennial belonging to the genus *Passiflora*. It can be found in the interior or edges of dense forests and shrublands, where vertical growth is essential to reach the top and compete for light. During the transition from juvenile to adult vegetative phases, *P. cincinnata* demonstrates marked leaf heteroblasty, accompanied by the appearance of tendrils that will be present in posterior phytomers.

Molecular studies have shown that the development of tendrils in the Passifloraceae family is a modification of reproductive structures (Sousa-Baena et al., 2018b, 2018a). However, information about the molecular bases involved in the development and growth of this organ is still incipient. Zhang et al., (2014) demonstrated in tobacco that the expression of AP1 genes, a target of SPLs transcription factors, is restricted to reproductive tissues in this species. Later, Scorza et al., (2017) presented anatomical and molecular evidence that the tendril in *Passiflora* is a modification of the reproductive part, and it was found that AP1 was associated with the initial stages of tendril development in *P. edulis;* afterward, it was proposed that tendrils develop as an extension of the inflorescence main (Cutri et al., 2013; Hernandes-Lopes et al., 2019).

Our objective was to identify differences in tendril growth and development caused by the miR156/SPLs module as a pathway regulated by age.

MATERIALS AND METHODS

GENERATION OF *miRNA156* OVEREXPRESSION AND SILENCING LINES IN *P. cincinnata* SERTÃO FORTE.'

Seed inoculation

Seeds of *P. cincinnata* Mast. cv. BRS 'Sertão Forte' were kindly donated by Dr. Fábio Gelape Faleiro (EMBRAPA Cerrados, Brazil). Subsequently, to the mechanical removal of the seed coat, the decoated seeds were surface-sterilized in 70% ethanol, for 60 s, under sterile conditions in a laminar flow hood. Following, immersed in a commercial sodium hypochlorite solution - 2.0-2.5% active chlorine (SuperGlobo, Super Globo Química, Contagem, Brazil) plus two drops of Tween 80 (for each 100 mL of bleach solution) for 15 min. The material was rinsed thrice (for 30 s each) with sterile water. The decoated seeds were inoculated in an MS medium containing MS basal salts and vitamins (Murashige and Skoog, 1962) (PhytoTechnology Laboratories$^{®}$, Kansas, USA), 0.01% (w/v) myo-inositol (Sigma Che, Co. St Louis, USA), 3.0%

(w/v) sucrose (PhytoTecnology Laboratories®), and 0.6% (w/v) agar (Plant TC Micropropagation Grade from *Gelidium* sp.; PhytoTechnology Laboratories®). The pH of the medium was adjusted to 5.8 ± 0.05 prior to the addition of the gelling agent and autoclaved at 121 °C, 108 kPa, for 20 min.

Approximately ten seeds were inoculated in each bottle (250 mL capacity) containing 50 mL of medium. The bottles were closed with polypropylene caps with two holes (10 mm) covered with 0.45 µm porous membranes (Milliseal®, AVS-045 Air Vent, Japan) to optimize gas exchange. Seeds were kept for 15 days in the dark, at 25 ± 1 °C, followed by 15 days under a photoperiod of 16 h and intensity of 50 ± 3 µmol m^{-2} s offered for white LEDs. Lamps.

Preparation of Agrobacterium tumefaciens

Cultivation of *A. tumefaciens* strain AGL-1 with independent miR156 silencing and overexpression constructs p35S: HygR pUbi10-STTM156-AtuOCS pMAS-CRT1 and p35S: HygR pUbi10-MIR156a-AtuOCS pMAS-CRT1 were cultivated in liquid LB medium containing 50 mg L^{-1} rifampicin (Aventis Pharma Ltda, Suzano, Brazil) and 100 mg L^{-1} kanamycin (PhytoTechnology Laboratories®) and carbenicillin (Gibco, Grand Island, NY, USA) for 48 h at 28°C and 120 rpm. The inoculum was used when the bacterial culture's optical density (OD_{600nm}) reached values of 0.45-0.5. Then it was centrifuged at 3500 *g* for 10 min. The pellet was resuspended in MS liquid medium, supplemented with 30 g L^{-1} of sucrose and myo-inositol for an OD600 nm of 0.3-0.5. Ten minutes before inoculation, sterile acetosyringone (Acros Organics, Morris Plains, NJ, USA) was added to the bacterial suspension.

Inoculation of explants and in vitro regeneration

The protocol for transforming hypocotyls in *P. cincinnata* was established in our research group (unpublished). Thirty days-old seedlings after in vitro germination (15 days dark/15 days light) were used as a source of explants to obtain the planned lines. Hypocotyls were sectioned transversally in segments of 0.8 - 1.0 cm in the suspension of *A. tumefaciens*, and after 15 min, the excess suspension was discarded. The explants were inoculated in the regeneration medium composed of MS salts and vitamins supplemented with 3.0% (w/v) sucrose, 100 mg L^{-1} myo-inositol, 6-benzyl amino purine (BA; PhytoTechnology Laboratories®) 1 mg L^{-1} and 100 μM acetosyringone (Sigma Chem. Co.), starting the co-culture stage when it was kept in the dark conditions at 25°C, for two days.

After, the explants were triple rinsed with sterile distilled water for 5 min each and subsequently with three washes with liquid MS medium containing 300 mg L^{-1} of Timentin (PhytoTechnology Laboratories®) and 250 mg L^{-1} Cefotaxime (PhytoTechnology Laboratories®). Subsequently, it was transferred to the MS-based selective medium, supplemented with BA 1 mg L^{-1}, Hygromycin (PhytoTechnology Laboratories®) at 6 mg L^{-1}, Norflurazon (Sigma Chem. Co.) at 3.8 mg L^{-1}, and Timentin 300 mg L^{-1}. Explants were kept at 25 ± 1 °C under a photoperiod of 16:8 h and irradiance of 50 ± 3 μmol m^{-2} s^{-1} for six weeks, and every 15 days selective culture medium was changed.

In the elongation phase, well-developed shoots (above 0.5 - 10 mm) were transferred to flasks containing hormone-free MS medium, supplemented with Hygromycin and Norflurazon for *in vitro* selection of transgenic lines. All GMO manipulations were performed in the Laboratório de Cultura de Tecidos II – Bioagro and in a GMO-adapted greenhouse following the CTNBio and CIBio-UFV biosafety rules (CQB 024/97, CTNBio nº 19, process number 01200.002610/9704).

GENOTYPING

Confirmation of insert introduction through Polymerase chain reaction(PCR)

To confirm the transgenic lines, genomic DNA was extracted from leaf primordia of acclimatized plants using the method described by Dellaporta et al. (1983). The presence of the insert in the transgenic lines was confirmed using vector-specific primers (Supplementary Table. 1). The lines were identified as NT: Untransformed, *OE::miR156*: overexpression of miR156, and *STTM156* silencing miR156.

Evaluation of the genetic stability of transgenic lines

The genetic stability of plants was evaluated at the Laboratory of Genetics and Biotechnology of the Institute of Biological Sciences (ICB) of the Federal University of Juiz de Fora (UFJF). The relative DNA content was obtained with a flow cytometer from leaf tissue of transgenic and non-transgenic lines. Young leaf core suspensions were prepared by chopping 25-

30 mg of young leaves with a disposable steel blade, as per Galbraith and Schendel, (1983), in a WPB isolation buffer (Loureiro et al., 2007). The suspension was filtered through a nylon membrane (30 µm mesh) to remove cell fragments and tissue debris and placed in polystyrene tubes. Afterward, 25 µl (1 mg mL^{-1}) of propidium iodide (Sigma Chem.Co.) and 5 µl (1 mg mL $^{-1}$) of Rnase (Amresco, USA) were added to stain the DNA. Samples were analyzed in a CytoFLEX 217 flow cytometer (Beckman Coulter, CA, USA). Three replicates were performed, and at least 10,000 nuclei were analyzed to quantify the fluorescence emission. As an internal standard, we used *Pisum sativum* (2C = 9.09 pg of nuclear DNA) (Dolezel et al., 1998). Histograms were generated and analyzed by CytExpert 2.0.1 program, and the DNA content (pg) (Supplementary Table. 2) was calculated according to Doležel and Bartoš (2005).

Analysis of gene expression by RT-qPCR

Leaf primordia ~0.5 cm were collected for RNA extraction. Approximately 100 mg of each sample were macerated in liquid nitrogen with an RNAse-free mortar and pestle. Then, the samples were transferred to 1.5 mL volume Eppendorf® tubes containing 1000 µL of Tri-Reagent® (Sigma) and 50 µL of a mixture of chloroform and isoamyl alcohol (24:1, v/v). The samples were homogenized by vortexing for 1 min, followed by incubation on ice for 5 min and centrifugation at 12,000 *g* for 20 min at 4 ºC. After centrifugation, the aqueous phase containing RNA (supernatant) was collected and transferred to an Eppendorf® type tube containing the same volume of isopropanol as the supernatant. Then, the mixture containing RNA was precipitated for one hour at -20 ºC and centrifuged for 30 min at 12,000 *g* and 4ºC. The pellets resulting from the centrifugation were washed in 1000 µL of 70% ethanol (RNAse-free) and centrifuged for 10 min at 12,000 *g* at 4 ºC. The supernatant was discarded, and the tubes were kept in a laminar flow chamber to remove an excess of 70% ethanol and dry the pellets. Soon after, the RNA was resuspended in 30 µL of water treated with diethylpyrocarbonate (DEPC, Sigma) and kept at -20 ºC. All samples were treated with DNAse I RNAse-Free (Ambion-Life Technologies), as the manufacturer recommends. RNA quantification was performed with the aid of NanoDrop ND-1000 (NanoDrop Technologies™) under an absorbance of 260 nm, and the integrity of the RNA samples was verified by 1.0% agarose gel electrophoresis (RNAse free). Subsequently, according to the manufacturer's instructions, single-stranded cDNAs were synthesized from 3.0 µg of total RNA using the SuperScriptTMII kit, First-Strand Synthesis System (Invitrogen™).

Finally, the cDNA was used as a template to amplify the coding sequence and quantify the genes of interest using different combinations of degenerate primers previously designed from the conserved domains of each gene (Supplementary Table. 1). For the design of the sequences of primers from different species available at the National Center for Biotechnology Information (NCBI) were used. RT-qPCR was performed in a CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD), using SYBR Green I (Bio-Rad Laboratories, Hercules, CA). The expression levels of target genes are presented relative to the expression of the constitutive Actin gene and calculated using the $2^{-\Delta\Delta Ct}$ method, according to (Livak and Schmittgen, 2001), consisting of three biological samples and two techniques repeat. Transcript levels of miR156 and SPL9 were analyzed.

PHENOTYPING

Acclimatization and transfer to the greenhouse

T0 plants regenerated *in vitro* were transferred to 150 mL disposable cups containing autoclaved Tropostrato® HT Hortaliças (Vida Verde Indústria e Comércio de Insumos Orgânicos Ltda, Mogi Mirim, Brazil) substrate. The initial acclimatization was carried out in the growth room with a photoperiod of 16 h, irradiance of 50 µmol m^{-2} s^{-1}, and temperature of 25 ± 1 °C for two weeks.

Then they were taken to a greenhouse at the Federal University of Viçosa (20º 45’ S, 42º 15’ W), Viçosa-MG. Then, the plants were transferred to 2-L polyethylene pots filled with horticultural substrate Tropstrato® and maintained under greenhouse conditions. The base fertilization and maintenance were made with Osmocote® Plus 15-09-12 + Micros (3-M) (ICL Co., USA), and maintenance fertilization was accomplished every 60 days. The tests consisted of four replications for each event; each experimental unit consisted of a plant.

Growth analyses

The phenological and structural differences between overexpression and silencing lines were analyzed. The variables evaluated were the height of the plant on the primary shoot, stem diameter between the third and fourth nodes and length of internode in the third, fourth, and fifth leaf (shootwards), and branching pattern. After, we analyzed the impact of miR156 levels on the formation and development of tendrils, identifying the position in which the first tendril developed.

In addition, tendril growth was measured every other day after emergence, and an increase in length of each tendril was recorded starting when the size was smaller than 1 cm avoiding contact with any surface to remain aligned. When it began to twist on its axis, the measurements were suspended. Also, the diameter of the base of 2, 6, 12, and 30 cm tendrils was determined.

Anatomical characterization of tendrils

For anatomical and histochemical evaluation, longitudinal cross-sections of the base and apical region of tendrils of 12 cm were performed. All samples were fixed in Karnovsky (Karnovsky, 1965). The samples were dehydrated in ethanol series and included in methacrylate (Historesin, Leica Instruments, Germany) for structural analysis. Cross-sections with 5 μm thickness were obtained on an automatic feed rotary microtome (RM2155, Leica Instruments, Germany). The sections were stained in toluidine blue (pH 3.2) for 15 min (O'Brien and McCully, 1981). Subsequently, the slides were mounted in Permount® SP15-500 (Fisher Scientific) and observed under a light microscope.

The images were captured in a light microscope (model AX70 TRF, Olympus Optical) with a U-photo system coupled with a digital camera (Spot Insightcolour 3.2.0, Diagnostic Instruments Inc.) and a microcomputer with the image capture software Spot Basic.

Hormonal profile

For quantification of endogenous hormone levels of IAA (indol-3-acetic acid), Zeatin, ACC (1-carboxylic acid-1-aminocyclopropane, reflects endogenous ethylene levels), ABA (abscisic acid), JA (jasmonic acid), and SA (salicylic acid), About 110 mg of tendrils of 12 cm of length from plants at 20 days of cultivation in the greenhouse, were macerated in liquid nitrogen and added 300 μL of extraction solution (methanol: Isopropanol: acetic acid 20:79:1) in a 1.5 ml microtube. Soon after, the samples were vortexed (4 times for 20 s), sonicated for 5 minutes, and kept on ice for 30 min. After centrifugation (13,000 *g*, 10 min at 4 °C), 350 μL of the supernatant was collected into a new microtube. The extraction process was repeated under the resulting pellet, and the supernatant was collected. LC-MS/MS (Agilent 1200 Infinity Series) coupled 276 to triple quadrupole QqQ-type mass spectrometer (6430 Agilent Technologies) was used for 277 hormone identification and quantification, according to (Napoleão et al., 2017).

Statistical analysis

The experimental design used was completely randomized. The data were submitted for analysis of variance using the software R® version 7.7, and Student's t-test compared the means at a 5% probability of error level.

RESULTS

Confirmation of transgenic lines

The role of miR156 as a pathway of age regulation and development was studied in different organs of *P. cincinnata*. For this, we use constructs containing the 35S promoter. In total, were obtained six overexpressing and nine silenced lines. All the analyses of phenotyping/genotyping were conducted in T_0 transgenic lines, one overexpressing, and two silencing lines. PCR initially confirmed the introduction of the construction into the genome in the leaves of explants regenerated on a selective medium. The size of the amplicon corresponds to the expected size, which includes the hygromycin antibiotic resistance marker gene *HygR*, strong promoter 35S, and sequence of the gene of interest (Fig. 1a).

In acclimatized plants, it was possible to confirm that the lines conserved the ploidy level after the regeneration process, maintaining its size compared to the control (Fig. 1b). Moreover, it was possible to confirm the expression levels of miR156. One of its targets, SPL9 and miR164, was found in some of the regulatory pathways of miR156. While the overexpression line shows a higher level of miR156 transcripts, the silenced lines do not differ significantly from the control due to the strategy used to silence miRNAs. In contrast, the levels of SPL9, belonging to the miR156 target family, SPLs, were significantly lower in the over-expression line and higher than the control in the silenced lines. Similarly, miR164 transcript levels were maintained significantly higher in miR156-silenced lines. (Fig. 1c).

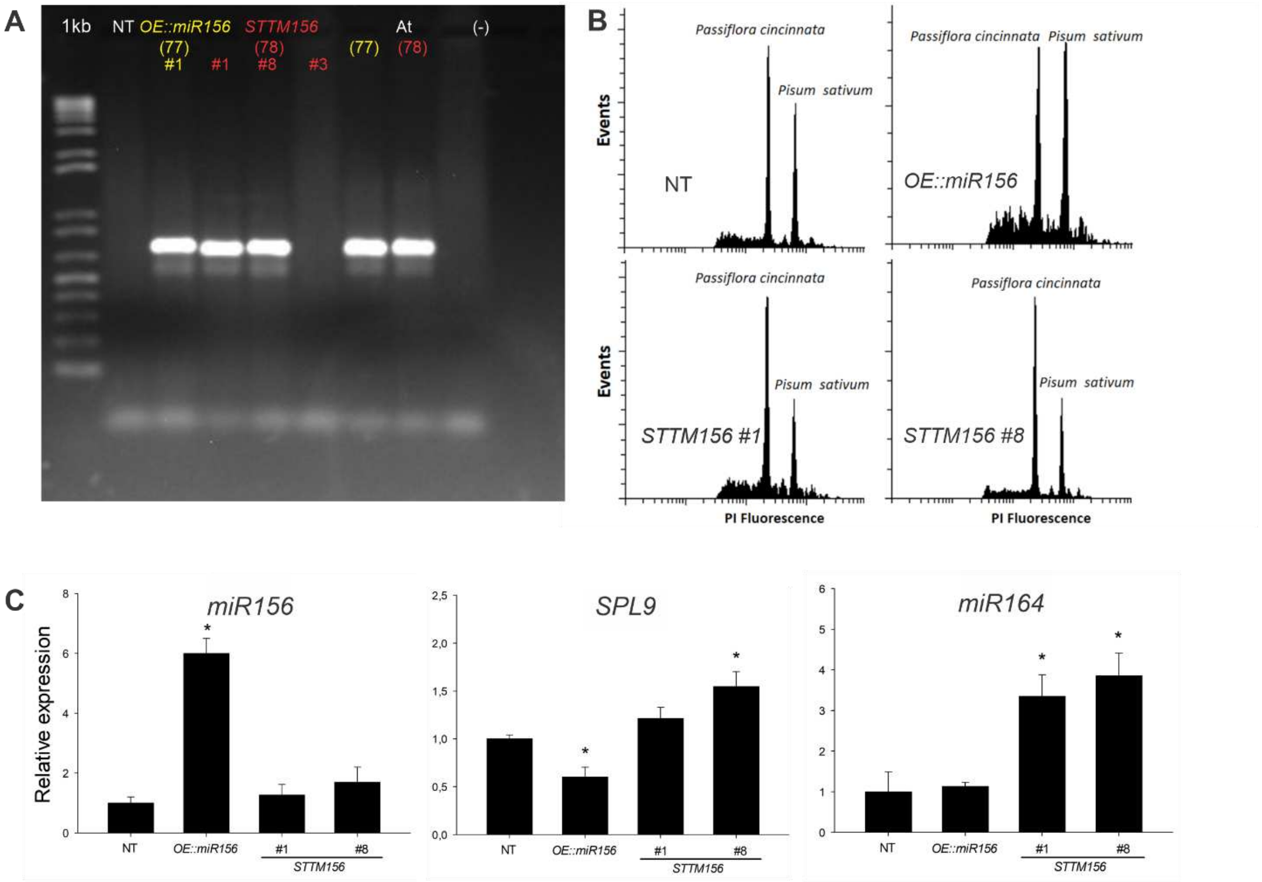

Fig. 1. Screening and confirmation of transgenic lines overexpressing and silencing the miRNA156. **A.** Amplification of NT (white), *OE::miR156* line (yellow), and *STTM156* #1 and #8 events (red). (-) corresponds to the negative control. *At* corresponding to *Agrobacterium tumefaciens* transformed with construction *OE::miR156* (77) and *OE::STTM156* (78). The PCR product has ~ 1800 bp. The primers were used to amplify the gene and the 35S promoter region. **B.** Flow cytometry for estimation of nuclear DNA content: Non-transformed (NT), overexpressing event *OE::miR156* and two silencing events *STTM156*, respectively. Histograms first and second peaks correspond to the *P. cincinnata* and the *Pisum sativum* internal standard (2C = 9.09 pg of nuclear DNA). **C.** *miR156/SPLs via-* related genes in one event of overexpressing and two of silencing. Asterisks indicate differences from NT according to Student's t-test ($P \leq 0.05$).

The differences in growth were marked. The overexpression line presented a smaller total height and shorter distance between internodes. On the other hand, there were significant differences in height, internodes length, and stem diameter when comparing overexpressing with NT and silenced lines. Also, we observed that the stem reached a smaller diameter when the plants had completed 120 days of growth in the greenhouse (Fig. 3).

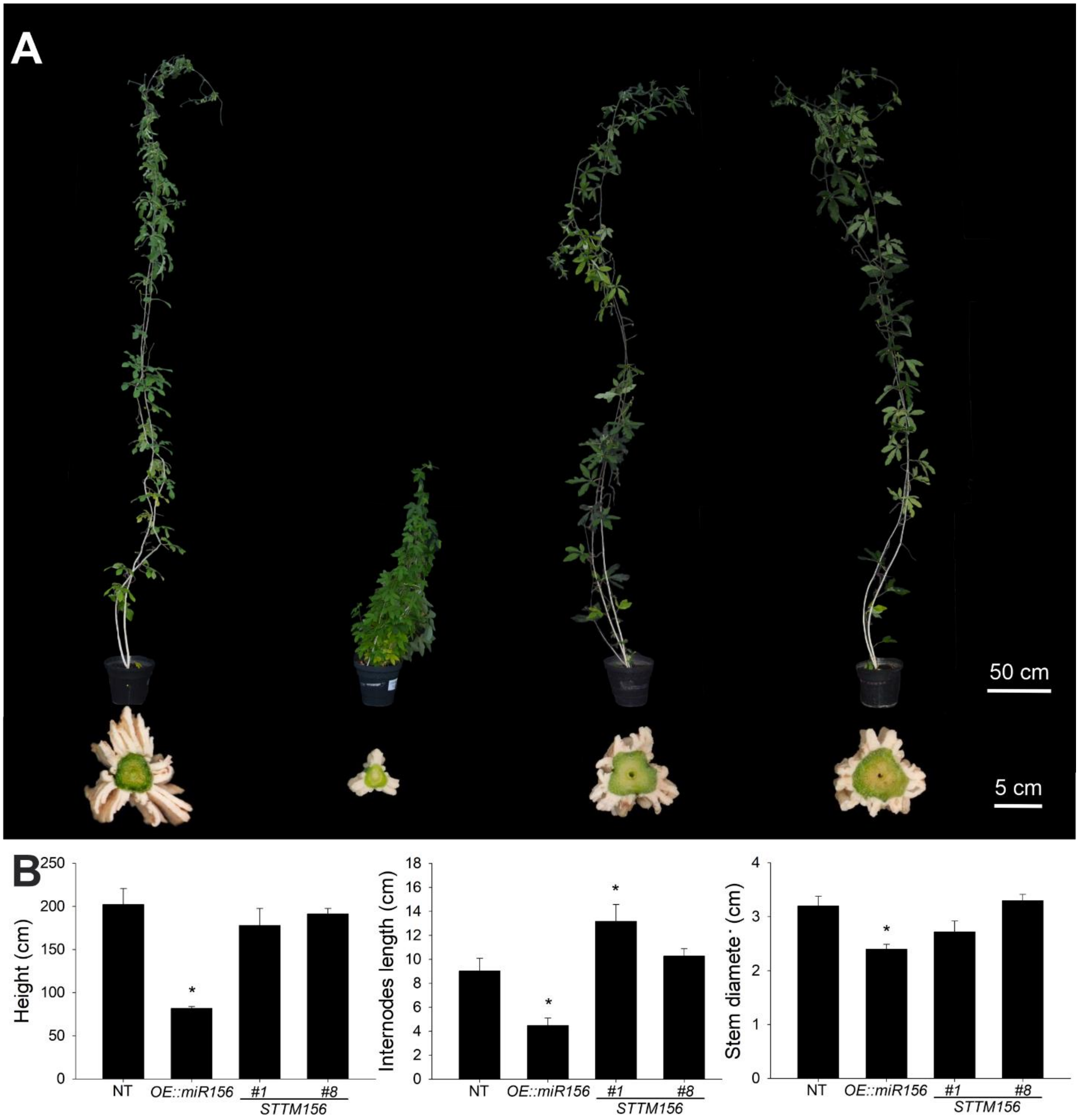

Figure 3. Growth and development are significantly affected by high levels of miR156 in *P. cincinnata*. A. Representative plants of NT, *OE::miR156,* and *STTM156* 120 days after

acclimatizing and cross-section of stem. B. Height, internodes length, and stem diameter measurements in plants of NT, *OE::miR156,* and *STTM156*. Values are presented as means ± SE (n=4). Asterisks indicate differences from NT according to Student's t-test (P<0.05).

Additional to differences in height, it was observed that the position of the first tendril was conserved in both the over-expression and silencing lines. Likewise, all plants started with trilobate leaves and then went through the transition, except in the over-expression line that remained in transition without reaching pentalobed. On the other hand, it was observed that lateral branching was increased in the over-expression lines showing less correlative inhibition. Interestingly, regardless of miR156 levels, the position of the tendril was not altered and occurred during the transition phase, i.e., before the development of the pentalobed leaves (Fig. 4).

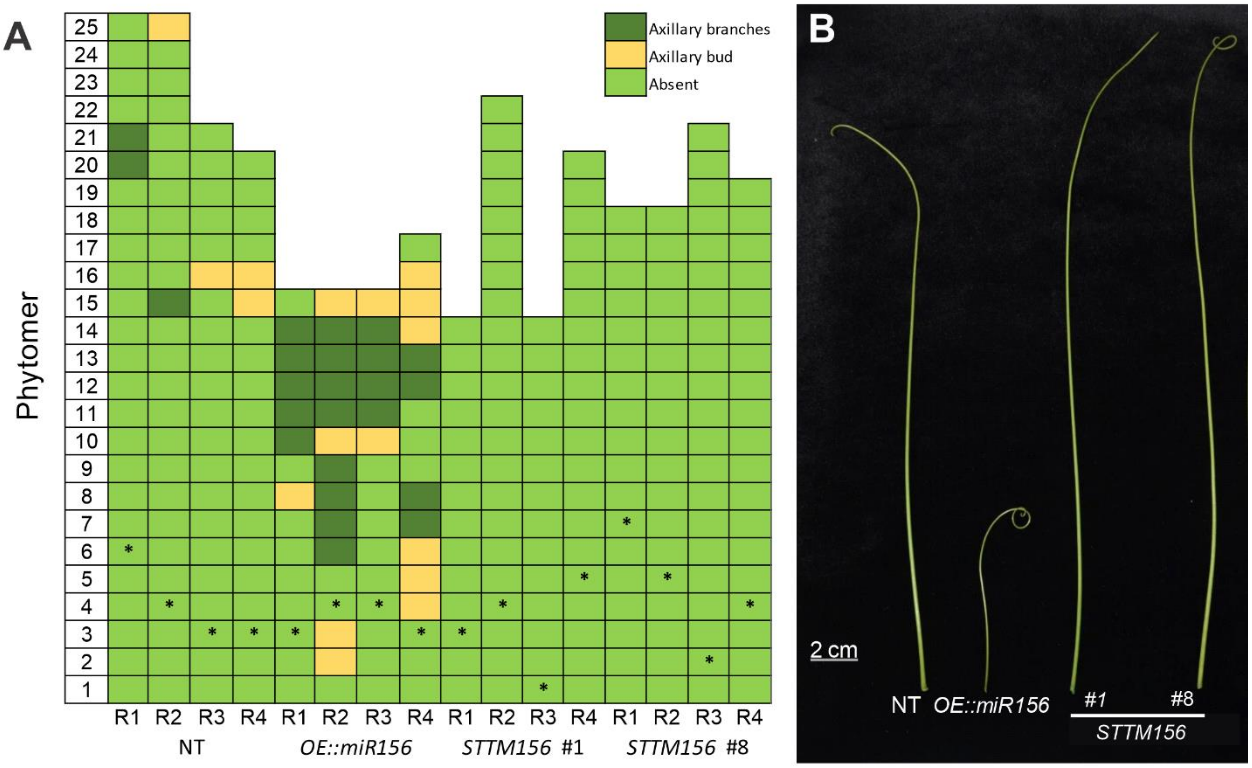

Figure 4. The branching pattern and growth of tendrils are affected mainly in the *OE::miR156* line. A. Branching pattern in plants of NT, *OE::miR156,* and *STTM156* 120 days after acclimatizing and representative transversal cross-section of stem. Asterisks indicate the position in which the first tendril was formatted.

The tendrils grew differentially, although, at the beginning of the development, no differences were observed, and growth was similar among all lines. When they reach approximately 12 cm, the tendrils of the overexpressing lines start to coil on their axis. In contrast, tendrils from NT and silenced plants only started to coil after reaching 30 cm (Fig. 5a). When comparing the diameter to the length; we observed that early in the development, the increase in diameter in the overexpression lines is reduced, and remains smaller until 12 cm, when it was possible to measure the extended tendril.

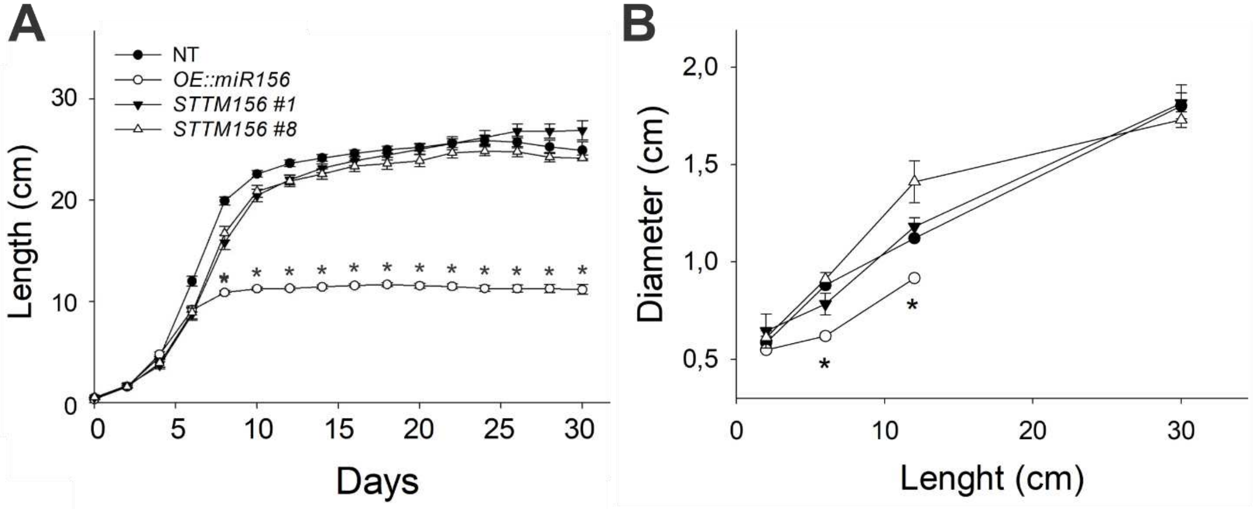

Figure 5. Tendril growth is regulated via miR156/SPLs. Overexpression represses the growth and development of tendrils. A. Growth curve of fifteen tendrils per plant. Values are presented as means ± SE (n=4). Asterisks indicate differences from NT according to Student's t-test (P<0.05). B. Representative fully developed tendril of NT, *OE::miR156,* and *STTM156.*

It was observed that the tendrils of 12 cm of NT and the silenced lines presented smaller and more compact cells in the apical and basal regions. In comparison, the overexpression line presented larger and more elongated cells. At the same time, less development of vascular bundles, as shown in the overexpression lines, and less tendril thickness. 6-7 layers of cells were observed in the apical region, and in the basal region, 8-10 layers in NT plants. In *OE::miR156* tendrils, 6-7 cell layers were observed in the apical region composing the cortical parenchyma and 6-8 layers in the basal region. The number of cell layers of the cortex was higher at the base, with 11-13 cell layers in the parenchyma, while at the apex, it remained 6-8 layers. These results indicate less cell division because tendrils with the same final elongation size have fewer and larger cell sizes (Fig. 6).

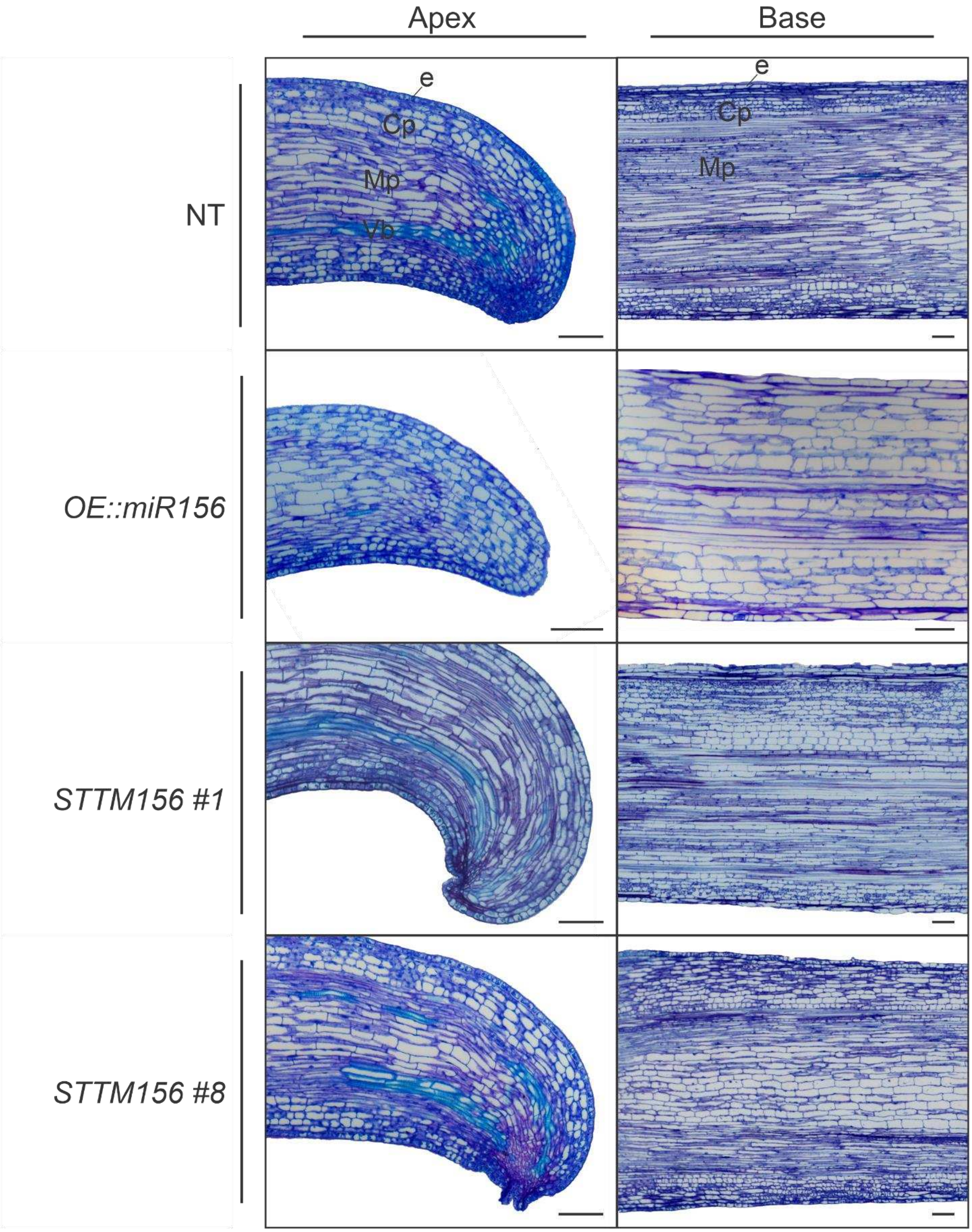

Figure 6. Tendril anatomy of *P. cincinnata* in non-transgenic and transformed overexpression and silencing lines. Cross-section of apex and base of tendrils of 12 cm length. Bars correspond to 200 µm at the apex and 100 µm at the base. e: epidermis; Cc: cortical parenchyma; Mpm: medullary parenchyma; and Vb: vascular bundle.

Endogenous hormone levels in 12 cm tendrils were assessed when growth was decreased in the *OE::miR156* line. A significant increase in MeJA and Brassinolide was observed in *OE::miR156*, while the silenced lines showed decreased ACC levels. No differences were observed in levels of auxin, zeatin, and ABA (Fig. 7).

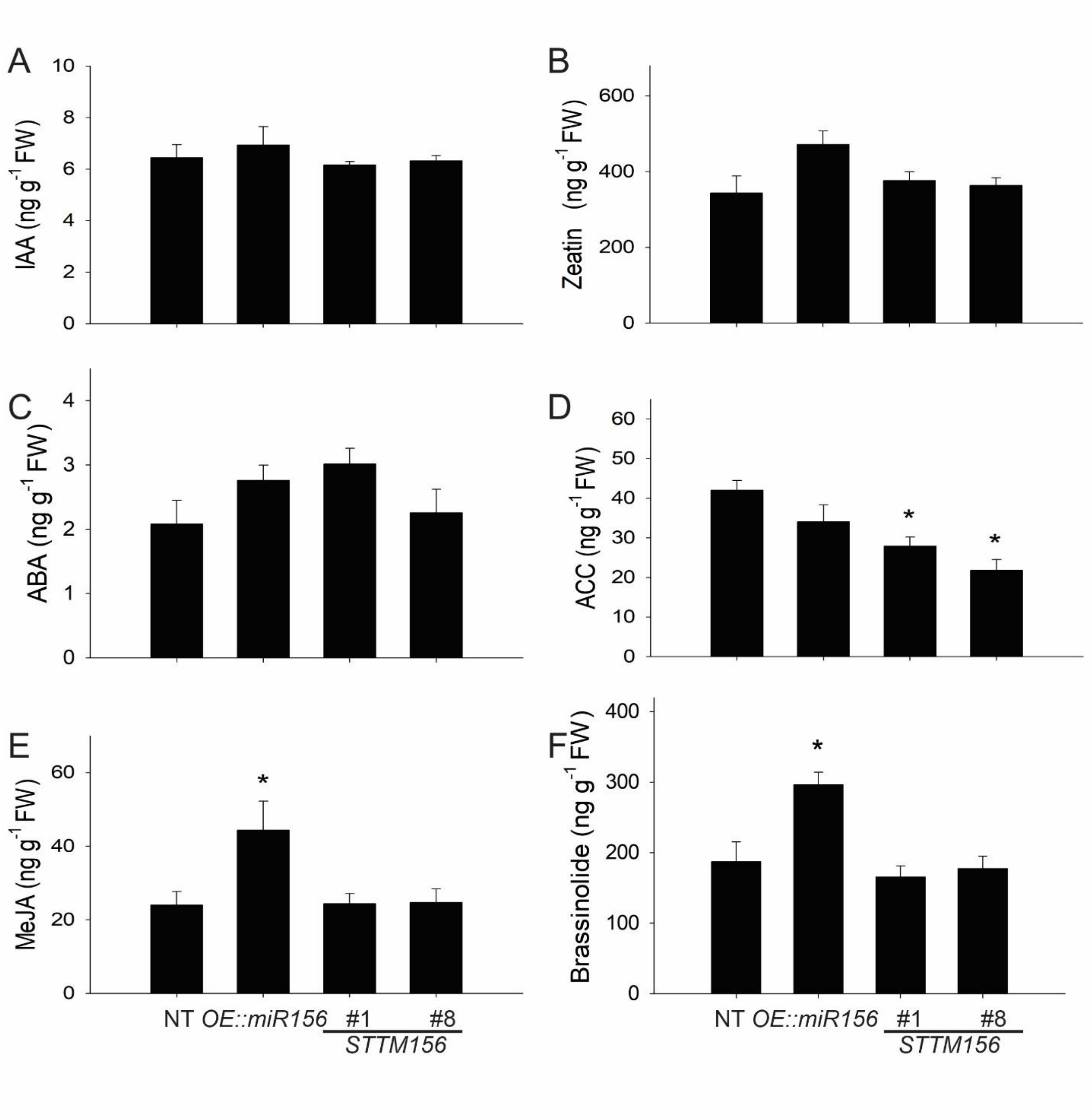

Figure 7. Hormone levels in tendrils of 12 cm in *P. cincinnata*. A. IAA (indol-3-acetic acid). B. Zeatin. C. ABA (abscisic acid) D. ACC (1-carboxylic acid-1-aminocyclopropane). E. MeJA (Metyl jasmonate). F. Brassinolide. Values were presented as means ± standard error of four biological replicates. Asterisks indicate differences from NT according to Student's t-test ($P \leq 0.05$).

DISCUSSION

As age advances, changes are expected to reflect progress in the phase transition, whether passively originated or regulated by active genetic programs. The transition from the juvenile to adult vegetative phase is accompanied by a decrease in miR156 expression, followed by an accumulation of SPLs, and an increase in miR172 levels, which leads to the adult vegetative phase and, subsequently, the reproductive phase (Wu et al., 2009; Yu et al., 2015b; Silva et al., 2019b). In *P. cincinnata*, one overexpression and two silencing lines for miRNA156 were confirmed and used in the experiments. After transformation, we confirmed the DNA content and expression levels of miR156, miR164, and SPL9. According to the transformation strategy, we found a significant increase in miR156 levels in the *OE::miR156* line and an increase in SPL9 levels, target of miR156 in the silenced lines. On the other hand, we evidenced increased levels of miR164 in the silenced lines.

At the molecular level, Soares (2020) confirmed that plants overexpressing miR156 presented higher levels of miR156 and lower levels of SPL9, as expected, and showed that miR156 levels are higher in 2-6 cm and decrease again when tendrils reach 12 cm. Similarly, a transcriptional analysis of tendril development in grapevine showed enrichment of genes associated with cell wall metabolism and lignification and negative genes associated with proliferation as development proceeds. Among the miR156 targets, SPL6, SPL9, and SPL13 were detected early in development, while SPL3 was most expressed in the mid-stage of development (Daz-Riquelme et al., 2014). SPL3 is an upstream activator of LEAFY, FRUITFUL, and APETALA1(Yamaguchi et al., 2009); these genes are expressed in the tendril meristem and, during development, are involved in leaf and plant maturation and lateral organ development (Hernandes-Lopes et al., 2019; Burko et al., 2013). In *P. edulis*, the organ with the highest expression of PeAP1 is the tendril (Scorza et al., 2017), indicating a possible role of this gene in tendril development. Thus, it is necessary to determine if there is a change in the levels of the mentioned genes, indicating that they have a role in tendril development controlled by the miR156/SPLs modulus as age advances.

In *Arabidopsis*, SPL9 participates in several processes, such as trichome distribution, leaf morphology, and induction of flowering (Wu et al., 2009; Yu et al., 2010; Jung et al., 2011; Yu et al., 2015a). Silva et al., (2019b) observed an increase in SPL9 transcript levels in adult leaves due to reduced miR156 expression during the heteroblastic process in *P. edulis*. In *Oryza sativum,* it was reported that overexpression of miR156 caused the reduction of six *SPLs* genes, but three other target genes showed no changes in expression. The *SPLs* gene family is known to have a high degree of functional redundancy in various processes during plant development (Wu et al., 2009).

Several research studies describe changes in plant architecture, maintenance of young traits, alteration of branching pattern, plastochron, and organ size, and flowering time according to miR156 levels (Zhang et al., 2015; Wang et al., 2016; Wang and Li, 2008). The lines overexpressing miR156 showed drastic changes in plant architecture, shorter height, a higher number of phytomere evidencing shorter internode length, and changes in lateral branching pattern suggesting correlative inhibition. Only one plant of NT, after 150 days, presented floral buds. However, plants overexpressing or silencing miR156 did not flower. Lines overexpressing *OE::miR156* in *P. edulis* showed reduced height and less stem development and control under heteroblasty and phase transition (Soares, 2020). These results are similar to those obtained in tobacco by Feng et al., (2016), where the effects of miR156 on growth and leaf shape were evidenced, showing plants of smaller size, more branched, and permanent foliar format juvenile characteristics in lines that are overexpressing miR156.

This behavior continued when analyzing tendrils. In the overexpression line, independent of the phytomere, the growth rate was similar at the beginning of the tendril development. All plants initially developed tendrils at the same growth rate. However, after approximately 12 cm, tendrils of *OE::miR156* started to coil on their axis without external stimulus, and the growth was stopped; while in plants NT and silenced lines, the growth continued with an extended tendril. Besides the size, the thickness is also smaller, making the tendrils less resistant and less able to bear their weight.

In this aspect, we must consider that *P. cincinnata* is a climbing species that grows in dense vegetation regions. It requires the timely development of tendrils to climb and compete for resources, mainly light. Young plants present less biomass and depend less on the tendrils for support; as the plant develops, the need for resources increases, and the height increment requires higher tendril resistance. (Soares, 2020) reported similar results in *P. edulis*, which showed structural changes with the lowest number of cell litters and the smallest final size of the tendril in plants overexpressing miR156. However, how the miR156/SPLs module control tendril development is unknown. thus, we decided to explore hormone levels when known to act as signaling molecules controlling growth and development through regulation of gene expression. It has been shown that this via be a mediator in the regulations involved in phytohormone crosstalk. Several studies have demonstrated the role of miR156 in this coordination; for instance, it was possible to demonstrate differences in the concentration of ACC, MeJA, and Brassinolide in tendrils. ACC was lower in the silenced lines, and MeJA and Brassinolide were higher in the *OE::miR156* line. IAA, Zeatin, and ABA showed no differences. In *Bryonia dioica* Jacq. was shown that it is possible to induce free coiling using a methyl-jasmonate cloud, while grape was shown to coil in response to JA in solution (Falkenstein et al., 1991).

Higher levels of MeJA in the *OE::miR156* line are likely involved with coiling in its axis, showing the tendrils at this stage, and this behavior was not observed in the silenced lines or the NT. Likewise, ethylene may be involved in the tendril coiling similar to that described by Jaffe, (1985) in pea tendrils. Brassinosteroids (BRs) levels were higher in the overexpression lines. BRs are involved in cell division, elongation, and differentiation throughout the entire plant life cycle; however, a possible explanation is a negative feedback, where high levels of BRs can lead to the inhibition of BR biosynthetic genes regulating homeostasis.

In the case of cytokinins, there were no differences between the lines, which confirms the results of (Zhang et al., 2015), who demonstrate that the regulation of miR156 is not at endogenous cytokinin levels but rather the response via B-type ARRs transcription factors altering sensitivity.

CONCLUSION

It was evidenced that the miR156/SPL pathway participates in multiple developmental processes in *P. cincinnata*. Our results indicate that changes in miR156 levels and, therefore, in SPLs triggered a regulatory network that caused lead to changes in plant heteroblasty and tendril development. At the same time, observed differences in tendril development suggest that the miR156/SPLs module does not play a central role in integrating age signals to promote tendril appearance. Still, it affects the growth and development of tendrils and directly affects successful plant support and climbing.

Further research around tendrils is needed because climbing plants are of great economic, ecological, and social importance, and several questions are left open at the end of this work. It is essential to determine if there are differences in the development of tendrils under growing conditions that simulate the natural environment to define how miR156 regulates their development and, consequently, the impact on ecological interactions.

REFERENCES

Burko, Y., Shleizer-Burko, S., Yanai, O., Shwartz, I., Zelnik, I.D., Jacob-Hirsch, J., Kela, I., Eshed-Williams, L., and Oria, N. (2013). A role for <i>APETALA1/FRUITFULL<7i> transcription factors in tomato leaf development. Plant Cell **25**: 2070–2083.

Chen, C., Zeng, Z., Liu, Z., and Xia, R. (2018). Small RNAs, emerging regulators critical for the development of horticultural traits. Hortic. Res. **5**: 1–14.

Chitwood, D.H. and Otoni, W.C. (2017). Divergent leaf shapes among Passiflora species arise from a shared juvenile morphology. Plant Direct **1**: 1–15.

Cutri, L., Nave, N., Ami, M. Ben, Chayut, N., Samach, A., and Dornelas, M.C. (2013). Evolutionary, genetic, environmental and hormonal-induced plasticity in the fate of organs arising from axillary meristems in Passiflora spp. Mech. Dev. **130**: 61–69.

Darwin, C. (1875). The movements and habits of climbing plants. Nature **13**: 65–66.

Daz-Riquelme, J., Martnez-Zapater, J.M., and Carmona, M.J. (2014). Transcriptional analysis of tendril and inflorescence development in grapevine (*Vitis vinifera* L.). PLoS One **9**: 92339.

Dolezel, J.., Greilhuber, J.., Lucretti, S.., Meister, A.., Lysak, M.A.., Nardi, L.., and Obermayer, R. (1998). Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann. Bot. **82**: 17–26.

Doležel, J. and Bartoš, J. (2005). Plant DNA flow cytometry and estimation of nuclear genome size. In Annals of Botany (Ann Bot), pp. 99–110.

Falkenstein, E., Groth, B., A, Mithöfer, A., and Weiler, E. (1991). Methyl jasmonate and α-linolenic acid are potent inducers of tendril coiling. Planta **185**: 316–322.

Feng, S., Xu, Y., Guo, C., Zheng, J., Zhou, B., Zhang, Y., Ding, Y., Zhang, L., Zhu, Z., Wang, H., and Wu, G. (2016). Modulation of miR156 to identify traits associated with vegetative phase change in tobacco (*Nicotiana tabacum*). J. Exp. Bot. **67**: 1493–1504.

Galbraith, C. and Schendel, D. (1983). An empirical analysis of strategy types. Strateg. Manag. J. **4**: 153–173.

Gianoli, E. and Molina-Montenegro, M.A. (2005). Leaf damage induces twining in a climbing plant. New Phytol. **167**: 385–390.

González-Teuber, M. and Gianoli, E. (2008). Damage and shade enhance climbing and promote associational resistance in a climbing plant. J. Ecol. **96**: 122–126.

Hernandes-Lopes, J., Sousa-Baena, M.S., Lemos, R.C.C., Corrêa, T.C.S., Van Sluys, M.A., and Melo-de-Pinna, G.F. de A. (2019). Toward understanding inflorescence development and architecture in Passiflora: insights from comparative anatomy and expression of *APETALA1*. Am. J. Bot. **106**: 1173–1189.

Jaffe, M.J. (1985). Ethylene and other plant hormones in thigmomorphogenesis and tendril thigmonasty. Horm. Regul. Plant Growth Dev.: 353–367.

Jung, J.H., Seo, P.J., Kang, S.K., and Park, C.M. (2011). miR172 signals are incorporated into the miR156 signaling pathway at the *SPL3/4/5* genes in Arabidopsis developmental transitions. Plant Mol. Biol. **76**: 35–45.

Karnovsky, J.M. (1965). A formaldehyde glutaraldehyde fixative of high osmolality for use in electron microscopy. J. cell. Biol **27**: 137.

Khraiwesh, B., Zhu, J., and Zhu, J. (2012). Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. Biochim. Biophys. Acta **1819**: 137–148.

Kus, J. V., Zaton, K., Sarkar, R., and Cameron, R.K. (2002). Age-related resistance in Arabidopsis is a developmentally regulated defense response to *Pseudomonas syringae*. Plant Cell **14**: 479–490.

Liu, W., Yu, W., Hou, L., Wang, X., Zheng, F., Wang, W., Liang, D., Yang, H., Jin, Y., and Xie, X. (2014). Analysis of miRNAs and their targets during adventitious shoot organogenesis of *Acacia crassicarpa*. PLoS One **9**: e93438.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods **25**: 402–408.

Loureiro, J., Rodriguez, E., Doležel, J., and Santos, C. (2007). Two new nuclear isolation buffers for plant DNA flow cytometry: A test with 37 species. Ann. Bot. **100**: 875–888.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with Tobacco tissue cultures. Physiol. Plant. **15**: 473–497.

Napoleão, T.A., Soares, G., Vital, C.E., Bastos, C., Castro, R., Loureiro, M.E., and Giordano, A. (2017). Methyl jasmonate and salicylic acid are able to modify cell wall but only salicylic acid alters biomass digestibility in the model grass *Brachypodium distachyon*. Plant Sci. **263**: 46–54.

Nave, N., Katz, E., Chayut, N., Gazit, S., and Samach, A. (2010). Flower development in the passion fruit *Passiflora edulis* requires a photoperiod-induced systemic graft-transmissible signal. Plant, Cell Environ. **33**: 2065–2083.

O'Brien, T.P. and McCully, M.E. (1981). The study of plant structure : principles and selected methods.: 357.

Rubio-Somoza, I., Zhou, C., Confraria, A., Martinho, C., von Born, P., Baena-Gonzalez, E., Wang, J., and Weigel, D. (2014). Temporal control of leaf complexity by miRNA-regulated licensing of protein complexes. Curr. Biol. **24**: 2714–2719.

Scorza, L.C.T., Hernandes-Lopes, J., Melo-de-Pinna, G.F.A., and Dornelas, M.C. (2017). Expression patterns of *Passiflora edulis APETALA1/FRUITFULL* homologues shed light onto tendril and corona identities. Evodevo **8**: 3.

Silva, G.F.F. et al. (2019a). Tomato floral induction and flower development are orchestrated by the interplay between gibberellin and two unrelated microRNA-controlled modules. New Phytol. **221**: 1328–1344.

Silva, P.O., Batista, Di.S., Cavalcanti, J.H.F., Koehler, A.D., Vieira, L.M., Fernandes, A.M., Barrera-Rojas, C.H., Ribeiro, Di.M., Nogueira, F.T.S., and Otoni, W.C. (2019b). Leaf heteroblasty in *Passiflora edulis* as revealed by metabolic profiling and expression analyses of the microRNAs miR156 and miR172. Ann. Bot. **123**: 1191–1203.

Soares, R.J. (2020). Efeitos da superexpressão do microRNA156 sobre a morfofisiologia de

Passiflora edulis Sims. durante a transição de fase juvenil/ adulta.

Sousa-Baena, M.S., Lohmann, L.G., Hernandes-Lopes, J., and Sinha, N.R. (2018a). The molecular control of tendril development in angiosperms. New Phytol. **218**: 944–958.

Sousa-Baena, M.S., Sinha, N.R., Hernandes-Lopes, J., and Lohmann, L.G. (2018b). Convergent evolution and the diverse ontogenetic origins of tendrils in angiosperms. Front. Plant Sci. **9**.

Voinnet, O. (2009). Origin, biogenesis, and activity of plant microRNAs. Cell **136**: 669–687.

Wang, Y. and Li, J. (2008). Molecular basis of plant architecture. Annu. Rev. Plant Biol. **59**: 253–279.

Wang, Y., Li, X., Zhou, W., Li, T., and Tian, C. (2016). De novo assembly and transcriptome characterization of spruce dwarf mistletoe *Arceuthobium sichuanense* uncovers gene expression profiling associated with plant development. BMC Genomics **17**: 1–14.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.W., Weigel, D., and Poethig, R.S. (2009). The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell **138**: 750–759.

Wu, G. and Poethig, R.S. (2006). Temporal regulation of shoot development in *Arabidopsis thaliana* by miR156 and its target SPL3. Development **133**: 3539–3547.

Yamaguchi, A., Wu, M.F., Yang, L., Wu, G., Poethig, R.S., and Wagner, D. (2009). The MicroRNA-regulated SBP-Box transcription factor SPL3 is a Direct upstream activator of *LEAFY, FRUITFULL,* and *APETALA1*. Dev. Cell **17**: 268–278.

Yu, N., Cai, W.J., Wang, S., Shan, C.M., Wang, L.J., and Chena, X.Y. (2010). Temporal control of trichome distribution by microRNA156-targeted *SPL* genes in *Arabidopsis thaliana*. Plant Cell **22**: 2322–2335.

Yu, N., Niu, Q.W., Ng, K.H., and Chua, N.H. (2015a). The role of miR156/SPLs modules in Arabidopsis lateral root development. Plant J. **83**.

Yu, S., Lian, H., and Wang, J.W. (2015b). Plant developmental transitions: The role of microRNAs and sugars. Curr. Opin. Plant Biol. **27**: 1–7.

Zhang, J., Yan, G., Wen, Z., An, Y.Q., Singer, S.D., and Liu, Z. (2014). Two tobacco *AP1-like* gene promoters drive highly specific, tightly regulated and unique expression patterns during floral transition, initiation and development. Planta **239**: 469–478.

Zhang, T.Q., Lian, H., Tang, H., Dolezal, K., Zhou, C.M., Yu, S., Chen, J.H., Chen, Q., Liu, H., Ljung, K., and Wang, J.W. (2015). An intrinsic microRNA timer regulates progressive decline in shoot regenerative capacity in plants. Plant Cell **27**: 349–360.

SUPPLEMENTAL MATERIAL

Table 1. Primer sequences were used for confirmation of transgenic lines and analysis of gene expression.

Primer	Sequence 5’-3’
AthUbi10:R1	ACGCCGTTTATTACTGACTCGTCGACA
P35S short_F1	GGTGGAGCACGACACTCTGGTC
PeACT F	CCAGGCATTGCTGATAGGAT
PeACT R	ATTCTGCCTTTGCAATCCAC
PeSPL9_ JF_ F	GGTCTAACCCAAATCCCGCA
PeSPL9_ JF _R	AGAGACCAGTGTGTGTGATGAG
miR156_ALeicht _F	GCGGCGTTGACAGAAGAGAGT
miR156/7_ ALeicht_ RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAC GTGCTC
Reverse universal__JF	GTGCAGGGTCCGAGGT
miR164	

Table 2. Averages DNA content, determined by flow cytometry, in plants of *P. cincinnata* NT, *OE::miR156* and *STTM156*

	DNA content (pg)	CV%
NT	3.10	2.812
OE::miR156	3.11	3.014
STTM156 #1	3.17	2.157
STTM156 #8	3.25	4.239

CHAPTER II

Role of miR156/SPLs pathway in growth and development of leaves and nectaries in *Passiflora cincinnata* Mast. (Passifloraceae)

ABSTRACT

Extrafloral nectaries are glands commonly described in passionflower plants. These secretory structures play an essential role in mutualistic trophic interactions. The offer of exudates of different compositions depending on the species is a reward that favors the visit of animals that can act as protection against herbivore attacks or pollen dispersion. The genus *Passiflora* shows a wide variety of nectaries that vary in shape, position, and number for each species. Likewise, on *P. cincinnata,* nectaries are observed on the leaf margin and petiole. Morphological changes during the phase transition reflect the temporal development of the apical stem meristem. *cincinnata* presents trilobed leaves in the juvenile phase and pentalobed leaves in the adult phase; the role of age, heteroblasty, and nectaries are unknown. The miR156-SPLs pathways regulate phase transition, organ development, and stress tolerance, among other processes. Here, we explore the role of the age mediated by the miR156/*SPLs* module in leaf nectaries of *P. cincinnata* from both the margin and petiole. Our results suggest that the miR156/*SPLs* pathway regulates the phase transition in *P. cincinnata*, where *OE::miR156* prevents the phase transition, while *STTM156* promotes early phase transition.

Thus, *OE::miR156* has a smaller number of lobes, smaller number, and size of nectaries, and therefore reduced ant visitation, contrasting with NT where leaves showed high lobulation and higher nectaries number; lines silenced with medium lobulation also presented an intermedium nectar number. This suggests an indirect role of the miR156/*SPLs* module in the development and function of nectaries through the modulation of leaf development and the appearance of lobes in adult plants.

Keywords: *Passionflower,* miRNA, *SPLs*, leaf development, lobes.

INTRODUCTION

Nectaries are specialized glands that secrete substances composed of sugars and various other compounds. They are located in flowers (Floral nectaries - FN), as well as in leaves, petioles, corolla, or fruit (Extrafloral nectaries - EFN) and have been described as gland secretory of reward for pollinators and insects that can act in their defense when attacked by herbivores and pathogens (Nicolson et al., 2007). The close relationship between petiolar EFN activity and simulated herbivory methyl jasmonate (MeJA), or indirectly low R:FR ratio, has been demonstrated (Izaguirre et al., 2013). On the other hand, it has been suggested that the size and number of nectaries and the quantity and quality of nectar may affect the preference of ants as visitors to EFN (Apple and Feener, 2001).

In the leaves of *P. cincinnata*, the presence of EFN is observed from the juvenile vegetative phase to the adult phase, where the juvenile leaves are trilobed and, in the transition to the adult stage, pentalobed leaves appear with a bidentate central segment (Fig. 1), this change in leaf pattern is called leaf heteroblasty. It is observed in several species of the *Passiflora* genus (Chitwood and Otoni, 2017).

In *P. cincinnata,* EFN is located in the adaxial region of the petiole and abaxial region of the leaf margin from of juvenile phase (Pinheiro, 2007). During the transition from juvenile to vegetative adult, there are changes in EFN in terms of number and size, mainly in the leaf margins. Interestingly, these changes in EFNs accompany the process of leaf heteroblasty, which in turn was correlated with the miR156-SPL pathway in *P. edulis* (Silva et al., 2019b). Kwok and Laird, (2012) suggest that in *Vicia faba* L., the number of nectaries increases with age and is significantly higher when plants suffer severe damage, Leichty and Scott Poethig (2019) describe that the production of EFN of *Vachellia* is correlated with a decline in miR156/157. Despite evidence of miR156-SPL regulation in the development of structures such as 'Beltian' bodies and *Vachellia* EFNs (Leichty and Scott Poethig, 2019), the mechanism of this regulatory center in *Passiflora* leaf EFN development remains unexplored.

Regulations mediated by miRNAs are involved in phytohormone crosstalk. Hormonal activity may trigger changes in the phase transition through interaction with the miR156/*SPL* regulatory module (Manuela and Xu, 2020). However, the interaction between microRNA and hormones is complex, as both can regulate each other. Several studies have shown that microRNAs can interact directly or indirectly with hormones through transcription factors, biosynthesis genes, signaling, and hormonal responses (Liu et al., 2009; Curaba et al., 2014; Zhang et al., 2015). Modulating miR156/*SPL* levels promotes changes in various aspects of the leaves like leaf shape, trichomes density, number of veins, stomata density, and size and density of epidermal cells (Yu et al., 2015b; Feng et al., 2016; Nguyen et al., 2017; Lawrence et al., 2021b). On the other hand, the activity of hormones such as auxin and cytokinin are important to leaf development and can also regulate changes in leaf characteristics, such as morphology, development, and patterning and vascularization of stomata (Eviatar-Ribak et al., 2013; Le et al., 2014; Shwartz et al., 2016; Linh et al., 2018) and possibly the development of EFN.

Although several genes involved in the development of floral nectaries like *Crabs Claw, Stylish*, or *Blade-on-petiole* (Bowman and Smyth, 1999; Roy et al., 2017; Toriba et al., 2019) have been described, little has been explored about the developmental pathways of EFN, both in molecular, metabolic, physiological, and ecological terms. Thus, our objective here was to explore the role of the miR156/SPL module in the formation and development of nectaries in *P. cincinnata,* as well as its effect on ant visiting.

MATERIALS AND METHODS

Plant material

For the investigation of the role of the miR156/*SPL* module in the development of extrafloral nectaries of *P. cincinnata*, transgenic overexpression (OE) silenced (STTM156) lines, and non-transgenic (NT) plants described in the previous chapter were used in this experiment. Greenhouse-grown plants were watered daily, and the maintenance of fertilization was carried out every 15 days.

Anatomical characterization of extrafloral nectaries

For anatomical evaluation, longitudinal sections of the leaf blade nectaries and transversal sections of the petiole nectaries were performed. All samples were fixed in Karnovsky (Karnovsky, 1965), dehydrated in ethanol series, and included in methacrylate (Historesin, Leica Instruments, Germany). Cross sections with 5 µm thickness were obtained on an automatic rotary microtome (RM2155, Leica Instruments, Germany). The sections were stained in toluidine blue (pH 3.2) for 15 min (O'Brien & McCully, 1981). Subsequently, the slides were mounted in Permount® SP15-500 (Fisher Scientific) and observed under a light microscope. The images were captured in a light microscope (model AX70 TRF, Olympus Optical) with U-photo system, coupled to a digital camera (Spot Insightcolour 3.2.0, Diagnostic Instruments Inc.) and a microcomputer with the image capture software Spot Basic.

Hormonal profile

For quantification of endogenous hormone levels of IAA (indol-3-acetic acid), Zeatin, ACC (1-Aminocyclopropane-1-carboxylic acid), reflects endogenous ethylene levels), ABA (abscisic acid), JA (jasmonic acid), and SA (salicylic acid), 110 mg of expanding leaves (3rd apex-base phytomere) from plants at 20 d of cultivation in the greenhouse, were macerated in liquid nitrogen and added 300 µL of extraction solution (methanol: Isopropanol: acetic acid 20:79:1) in a 1.5 mL microtube. Soon after, the samples were vortexed (4 times for 20 s), sonicated for 5 min, and kept on ice for 30 min. After centrifugation (13000 *g*, 10 min at 4°C), 350 µL of the supernatant was collected into a new microtube. The extraction process was repeated under the resulting pellet, and the supernatant was collected. LC-MS/MS (Agilent 1200 Infinity Series) coupled to a triple quadrupole QqQ-type mass spectrometer (6430 Agilent Technologies) was used for hormone identification and quantification, according to (Napoleão et al., 2017).

Characterization of extrafloral nectaries

The number of extrafloral nectaries was determined in three leaves of different shoots per plant, using four plants for each line. There quantified by separating in trilobate and pentalobate leaves and differentiating between the petiole and leaf margin nectaries. The nectaries were photographed from the leaves of the fifth and sixth phytomer base to the apex under a stereomicroscope and measured for diameter (cm) and length x width (mm) using Image J software.

Ant visitation

To determine whether modulation of miR156 expression can influence the relationship of *P. cincinnata* with ants, we registered ant visitation in extrafloral nectaries following the methodology with adaptations of Izaguirre et al. (2013). Observations were performed at noon for ten days, using four plants from each row located randomly on the greenhouse bench. The observations were repeated thrice.

Statistical analysis

The experimental design used was completely randomized. The data were submitted for analysis of variance using the R® version 7.7 program and Student's t-test to compare the means at a 5% of probability.

RESULTS

Modulation of miR156 levels affects changes in leaf morphology and hormone levels associated with leaf development P. cincinnata

Analyzing leaf development and heteroblasty in *P. cincinnata,* we observe that NT plants start with trilobed leaves. Then, the leaf transition begins, forming the other two lobes, and approximately after six leaves in transition, the first pentalobate leaf appears, which characterizes the adult vegetative stage. Interestingly, the plants of the overexpression line did not present pentalobed leaves, remaining in transition (Fig. 1). However, silenced lines displayed leaf development in line with NT, with pentalobed leaves with deep sinus (Fig. 1).

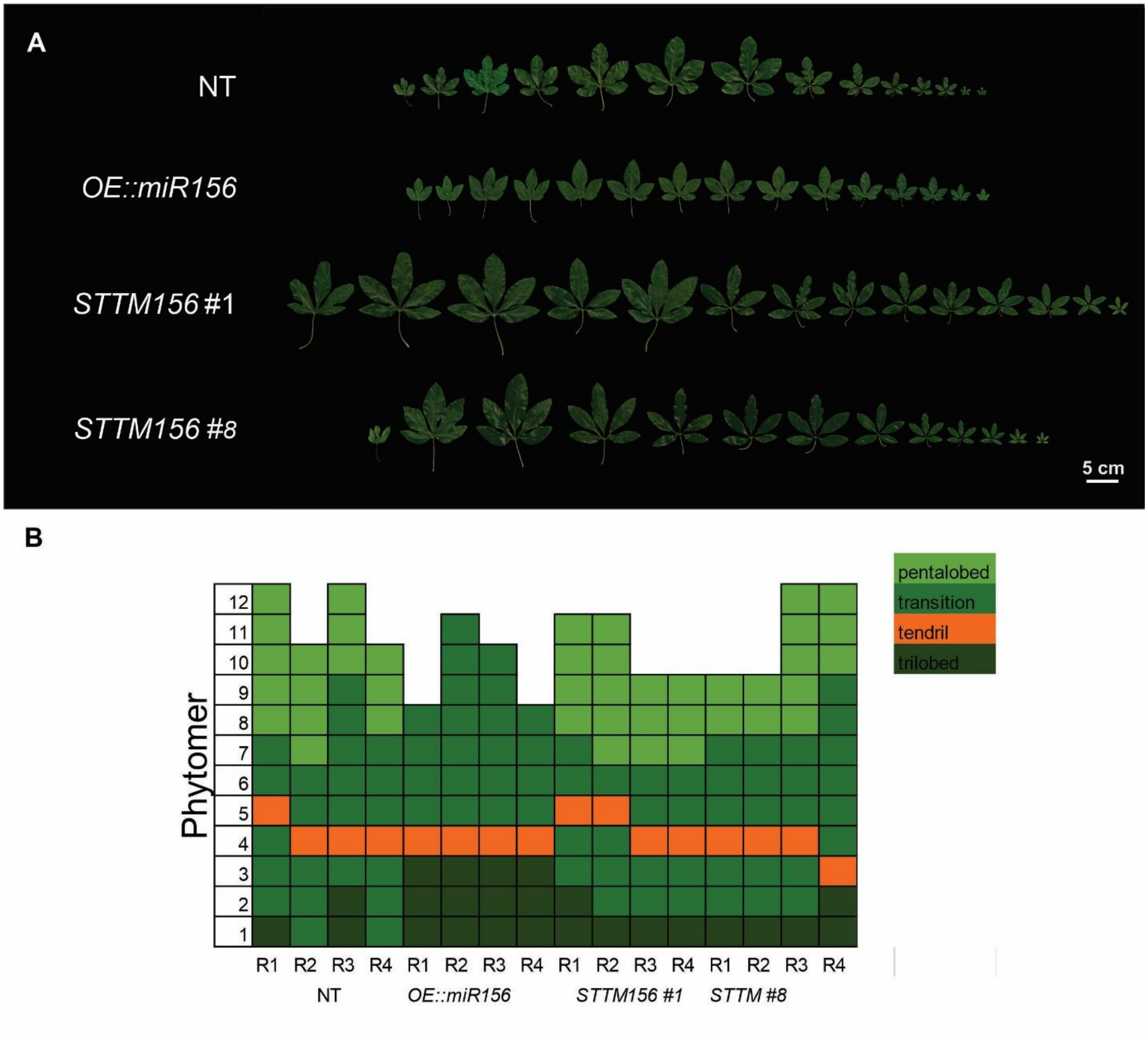

Figure 1. Overview of the heteroblastic process during leaf development from juvenile to adult in the vegetative phase of *P. cincinnata.* A. Representative leaves of NT, *OE::miR156,* and *STTM156* lines, respectively. Left to right, from older trilobed leaves in the base to adult pentalobed leaves in the apical. **B**. Phase transition map in *P. cincinnata* after plant pruning. Dark green corresponds to trilobed leaves, medium green to transitional, and light green to pentalobed leaves. Orange indicates the position where the first tendril appeared. It is evident that the relationship between

the miR156/SPL module and leaf development during the phase transition, where *OE::miR156* affected the formation of lobes, a characteristic described for adult plants in *P. cincinnata*.

Regarding the role of hormones in leaf development and the impact of manipulating miR156 levels on endogenous phytohormone levels, we found that IAA, zeatin, and ABA showed significantly lower levels in the *OE::miR156* line when compared to NT (Fig. 2A-C). ACC levels were higher in *STTM156* lines (Fig. 2D). Although MeJA levels did not show a significant difference between the genotypes, the *OE::miR156* line showed a lower level of this hormone (Fig. 2E).. Similarly, Brassinolide did not show significant differences; however, all the transgenic lines showed lower levels than NT (Fig. 2F).

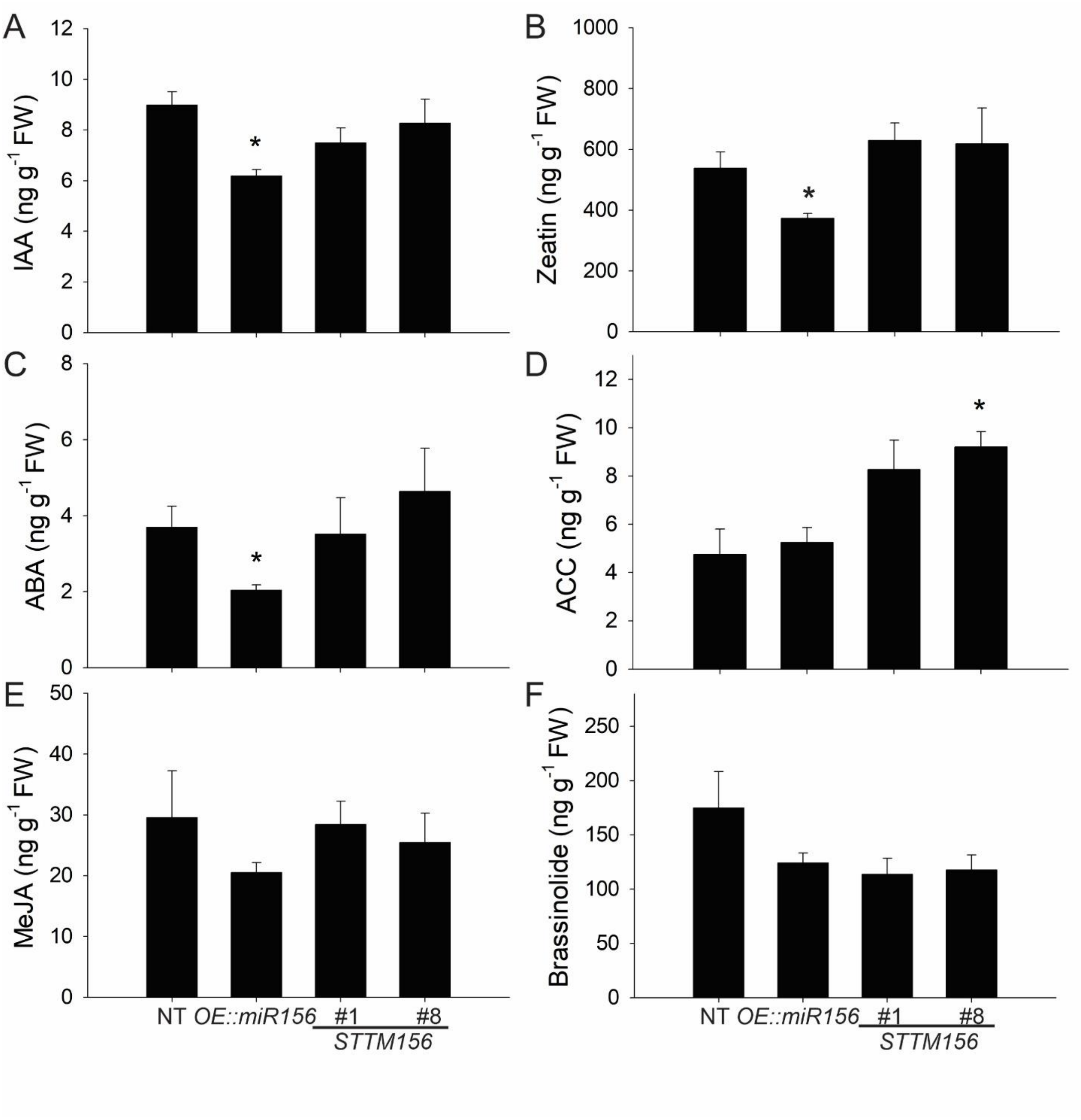

Figure 2. Hormone levels in leaf primordia of less than 1 cm in *P. cincinnata*. **A**. IAA (indol-3-acetic acid). **B**. Zeatin. **C**. ABA (abscisic acid). **D**. ACC (1-carboxylic acid-1-aminocyclopropane). **E**. MeJA (Metyl jasmonate). F. Brassinolide. Values were presented as means ± standard error of four biological replicates.

The development and growth of extrafloral nectaries in P. cincinnata depend on miR156 levels.

P. cincinnata presents EFN in the leaf margins and petioles. The number of petiolar nectaries was compared between the genotypes. All showed only one pair of EFNs occurring in parallel; there were no differences in the number of EFNs according to miR156 levels. Concerning the EFN on the margins in trilobed leaves, there were no differences in the number of EFN between genotypes. However, pentalobed leaves showed a significant decrease in nectaries number in foliar transition and pentalobed in the *STTM156* lines compared with NT. While pentalobed leaves of NT plants presented around ten pairs of nectaries in the margins leaf, the *OE::miR156* line presented approximately five, and the *STTM156* ones around ten nectaries per leaf (Fig.3). These results demonstrate that differences in the number of nectaries at leaf margins are dependent on miR156/*SPLs* levels.

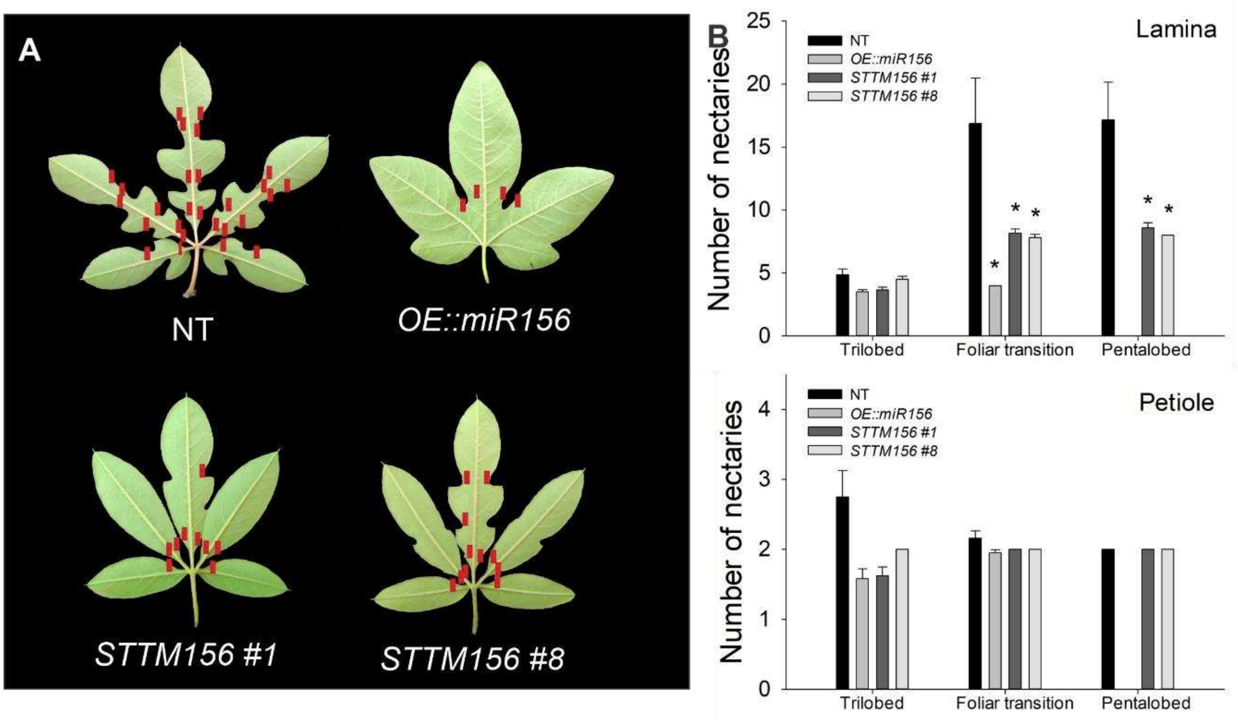

Figure 3. The number of extrafloral nectaries in different miR156 expression levels in *P. cincinnata*. **A**. Position of nectaries in NT, *OE::miR156,* and *STTM156* lines. **B**. Number of nectaries in lamina and petiole. Values are presented as means ± SE (n=4). Asterisks indicate differences from NT according to Student's t-test ($P \leq 0.05$).

The growth and anatomical pattern of laminar and petiolar nectaries were affected by miR156 modulation

In NT plants, differences were observed in the size and diameter of the NEF comparing the trilobed and pentalobed leaves, both in the blade and in the petiole, being more extensive in the pentalobed leaves as expected. At the same time, miR156 super-expressing lines were characterized by smaller, thinner nectaries with fewer trichomes. It also observed a greater density of trichomes in STTM lines (Fig. 4-5).

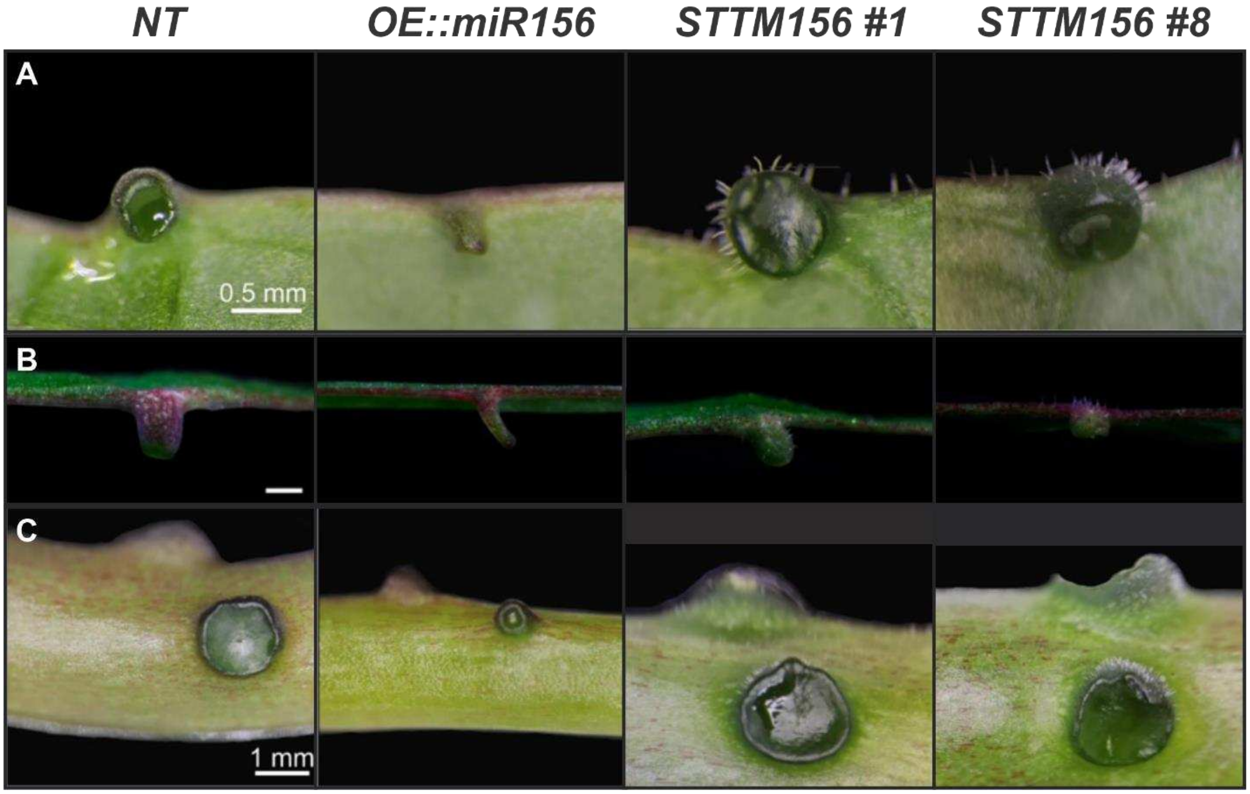

Figure 4. Growth and morphology of extrafloral nectaries in *P. cincinnata* are affected by the miR156/*SPLs* module. **A**-**B**. Representative laminar nectaries in the top and side view. **C**. Representative petiolar nectaries.

When comparing the trilobed leaves, it was observed that the laminar nectaries present smaller diameters and smaller sizes (length X width) in *OE::miR156* plants that NT. In contrast, the *STTM156* lines presented larger nectaries (Fig. 5A-B). Surprisingly, in the pentalobed leaves, the pattern is not repeated, and the two silenced lines had LxA lower than NT, while the diameter did not show significant differences. Only NT differed in both parameters when comparing trilobed and pentalobed leaves within the same genotype.

In diameter parameters, petiolar EFNs in trilobed leaves were significantly lower in the *OE::miR156* line and higher in the *STTM156 #8* line compared to NT (Fig. 5C-D). About LxA, the *OE::miR156* line did not differ from NT, while the STTM lines were significantly greater. In the pentalobed leaves, there were no significant differences in diameter size between the *STTM156* and NT lines; however, *STTM156#1* differed in LxA. When comparing trilobed and pentalobed leaves within the same genotype, the diameter, and LxA were significantly higher in NT and *STTM156#8* pentalobed leaves.

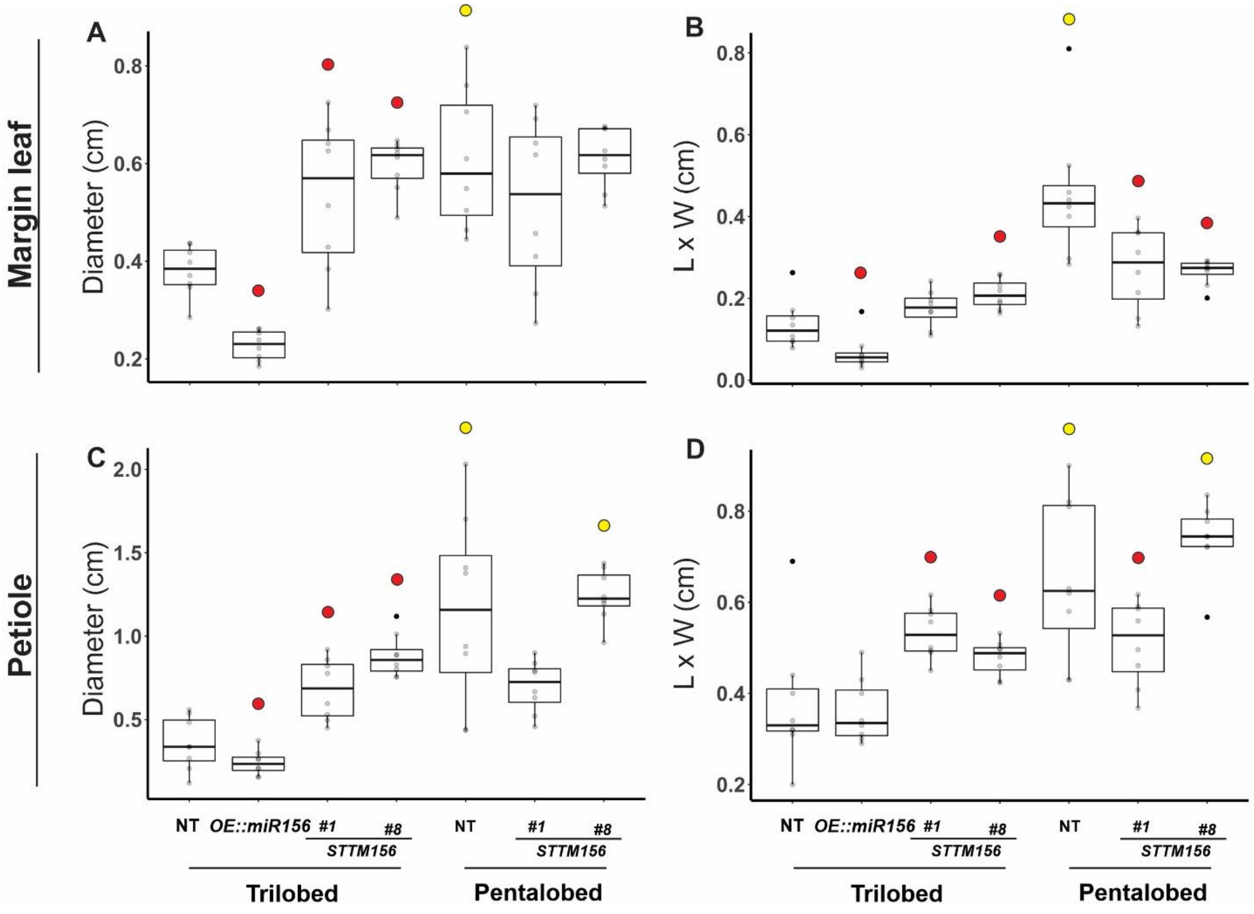

Figure 5. Extrafloral nectary size in *P. cincinnata* depends on the stage of leaf development and miR156 levels. **A** and **B** correspond to nectaries from the blade leaf; **C** and **D** to nectaries from the petiole. Points above the boxes indicate P ≤ 0.05 in Student's t-test. Red dots compare lines with

the respective control within each group, trilobed and pentalobed. Yellow dots compare trilobed and pentalobed leaves in the same line.

EFNs of margins and leaf petiole in pattern *P.cincinnata* is a concave structure, and there were no transgenic genotype modifications. In NT plants, the laminar EFN epidermis comprises two layers of juxtaposed cells. Below the epidermis, the secretory parenchyma with slightly isodiametric cells with large vacuoles and dense contents. The subnectariferous parenchyma comprises more extensive and isodiametric shape cells with significant vacuolated vascular connections that can be seen throughout the subnectariferous parenchyma (Fig. 6). In the *OE::miR156* line, both the nectaries of the leaf margins and the petiole showed reduced size, mainly thickness. The nectaries of the blades presented a uniseriate epidermis with large rounded cells; below the epidermis, more spherical cells with low-dense vacuolar content were observed compared to NT. Elongated cells make up the region distal to the epidermis. In silenced plants, nectaries at the leaf margin form a calyx, with the epidermal cells multiplying into many layers of secretory cells. The modification described can modify the nectary structure and the nectar elimination mechanism.

The structural organization of petiolar EFNs did not show wide differences between the genotypes, despite *OE::miR156* presenting a thinner structure. Petiolar EFNs presented the epidermis with juxtaposed cells. In many *Passiflora* species, the parenchymal tissue of petiolar EFNs is subdivided into nectariferous and subnectariferous (Simonetti and Devoto, 2018; Silva et al., 2017). We found the same pattern in *P. cincinnata*, whose nectariferous parenchyma cells were partially isodiametric with idioblasts, mainly in NT plants. Vascularization occurred mainly in the subnectariferous parenchyma.

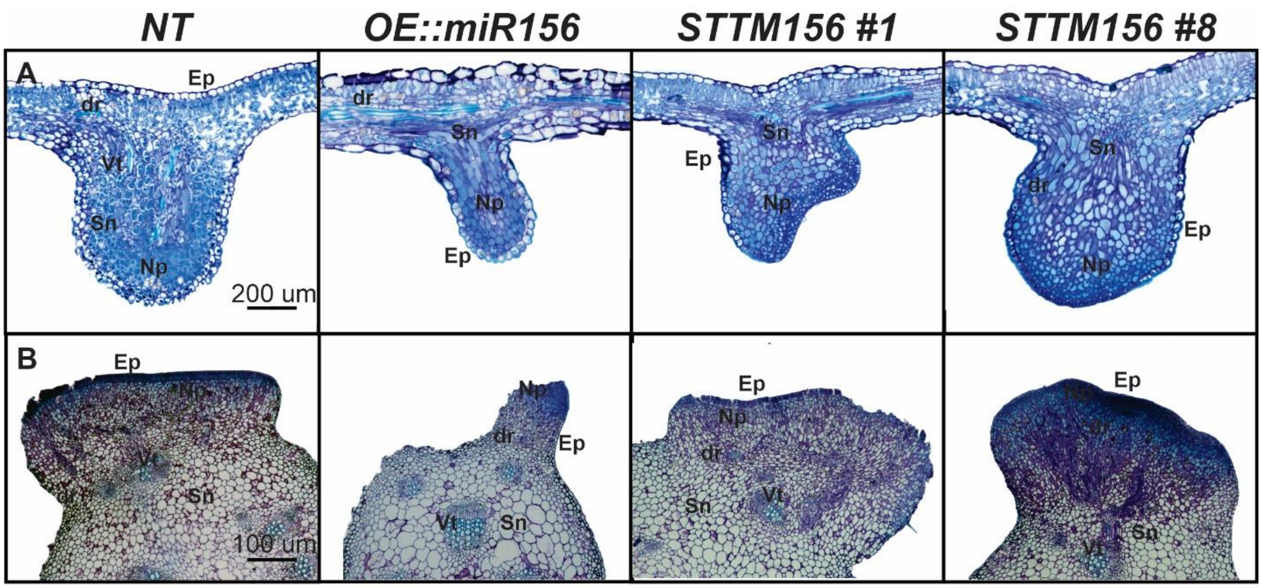

Figure 6. Development of extrafloral nectaries in *P. cincinnata* is affected by miR156/*SPLs* via. **A**. Cross section of petiolar nectaries. **B**. Transversal cross-section of laminar nectaries. (Ep: epidermis; dr: druse; Np: nectary parenchyma; Sn: subnectary parenchyma; Vt: vascular tissue). Scale bars: A: 200 µm; B: 100 µm.

Alterations in growth and development in the nectaries may be affecting the ecological relationships of P. cincinnata.

After verifying a habitual visitor to the plants, the number of ants was counted at the time of increased activity, and later calculated the number per branch. The ants visiting were identified as of the genus *Brachymyrmex* during the experiment. The visitation of ants in the *OE::miR156* plants was low. In contrast, the *STTM156* plants showed a less drastic decrease in visitation compared to NT (fig. 7). This result can be associated with reducing the number of nectars in the leaves of *OE::miR156* and *STTM156* plants or even lower exudation or nectar quality. Although at the time of evaluation, the supply of nectar was consistently observed in the plants *OE::miR156*. However, an investigation of the quantity and quality of nectar is necessary for plants that overexpress or silence miR156. Our results indicate that the mutualism between *P. cincinnata* and ants may be age-dependent, similar to the results found in Acacias plants that offer food and shelter as rewards for ants of *Pseudomyrex* genus being suggested that the miR156/SPL pathway is critical in the regulation of these changes (Leichty & Poethig, 2019).

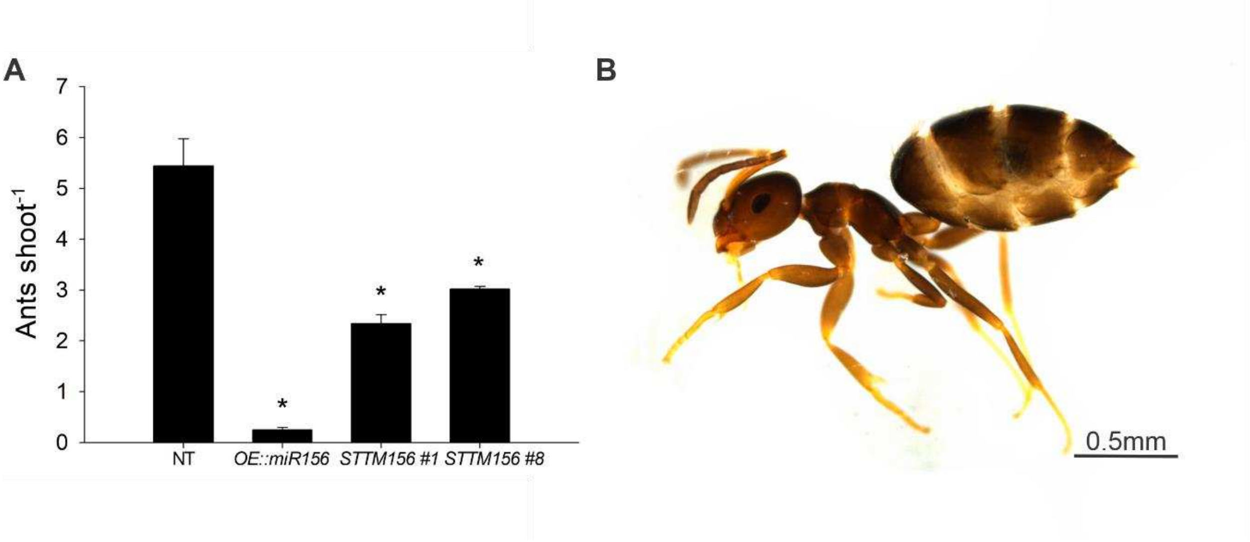

Figure 7. The preference for Brachymyrmex is altered when miR156 is overexpressing or silencing. **A.** Ants visitation. Values are presented as means ± SE (n=4). Asterisks indicate differences from NT according to Student's t-test ($P \leq 0.05$). **B**. *Brachymyrmex* spp. Ant species identified in plants of *P. cincinnata*.

DISCUSSION

During the juvenile phase, the levels of miR156 are high and decrease as the plant reaches the adult vegetative phase; at the same time, the expression levels of *SPLs* increase. The dynamic expression of miR156 and its target genes, *SPLs* promotes morphological and physiological changes in plants (Feng et al., 2016; He et al., 2018; Lawrence et al., 2021a); however, these changes during the transition of phases are species-dependent.

P. cincinnata presents marked leaf heteroblasty; in the juvenile vegetative phase, the leaves are trilobed, and the number and deeply of lobes on the leaf gradually increase during the transition until form five lobes in adulthood. Plants with miR156 overexpression did not present pentalobed leaves, remaining trilobate and rounded shape throughout this study. On the other hand, in *STTM 156* lines, the production of pentalobed leaves was early compared with NT. These results suggest that leaf heteroblasty *in P. cincinnata* is regulated by the miR156/*SPL* pathway, as reported *for P.*

edulis, whose change in leaf morphology was associated with the decrease of miR156 expression levels and an increase in miR172 levels and *SPL9* (Silva et al., 2019b). The modulation of the levels of miR156 in *Arabidopsis*, *Nicotiana tabacum*, *Populus*, and *Solanum lycopersicum* affected at least one characteristic of the leaf, such as vein density, leaf shape, production of trichomes (Wang et al., 2011; Silva et al., 2014; Feng et al., 2016; Lawrence et al., 2021b, 2021a), evidence that some leaf aspects may be under direct or indirect regulation of the miR156/*SPL* module. In *Arabidopsis*, *SPL9* is the main determining gene of leaf morphology changes as the plant ages. *SPL9* can form a complex *with TEOSINTE BRANCHED/CYCLOIDEA/PCF* (*TCP*), preventing it from forming a dimer with *CUP-SHAPED COTYLEDON* (*CUC*), providing the increase of serration and complexity of the leaves (Rubio-Somoza et al., 2014).

In *P. cincinnata*, in addition to changes in leaf morphology, the modulation of miR156 also affected the endogen levels of IAA, ZEA, ABA, and ACC. The level of IAA and ZEA was lower in line *OE::miR156*, which maintains the juvenile characteristics of the leaves. On the other hand, the *STTM156* strains did not present significant changes in the levels of these hormones.

miR156 and SPL can regulate biosynthesis genes of hormones, receptors, and transcription factors involved in signal transduction (Mao et al., 2017; Yu et al., 2012; Zhang et al., 2015). In addition, the signaling and response of IAA, GA, and ABA may directly or indirectly affect the miR156 levels or *SPLs* genes (Marin al., 2010, Tian et al., 2020; Manuela and Xu, 2020). Silva et al. (2019) demonstrated that IAA levels increased as the expression of miR156 increased and that high levels of IAA reflect the formation of lobes in leaves and the gain of adult characteristics in *P. edulis.*

In studies with shoot bud regeneration, it was demonstrated that SPL2, SPL9, and SPL10, all targets of miR156, physically interact with type-B transcription *factors ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) modulating the response but not the cytokinin level, reducing regenerative capacity (Zhang et al., 2015). In contrast, our results have shown that miR156/*SPL* can also modulate cytokinin levels. Mutant *Arabidopsis* plants in cytokinin receptor ARABIDOPSIS HISTIDINE KINASE2 (*ahk2 ahk3*) and overexpression of cytokinin degradation enzyme CKX1 (CKX1ox) with low cytokinin levels showed juvenile leaf phenotype and prolonged juvenile phase, in addition to levels of miR156 in the CKX1ox genotype. On the contrary, mutant plants for CKX1 (ckx3,4,5,6) and AHK2 with function gain showed higher levels of cytokinin and early promotion of transition to adulthood (Werner et al., 2021).

Considering the role of auxin and cytokinin in leaf development (Shwartz et al. 2016), crosstalk between these hormones and miR156/*SPL* may contribute to the differences in leaf morphology between transgenic lines and other leaf components, such as EFNs (Fig. 7 A). Genes related to hormonal responses have also been described as functional in developing floral nectars, for example, AUXIN RESPONSE FACTOR6 (ARF6) and ARF8, whose double mutant arf6/8 showed a phenotype without floral nectars, suggesting that auxin and possibly other hormones may mediate nectary development pathways. In addition, jasmonates and gibberellins have been described as potential regulators of nectar production and secretion (Roy et al., 2017); however, the relationship between miR156/*SPL* and the transduction of hormonal signals in the development of EFNs requires further studies.

ABA is a hormone related to abiotic stress, which controls several aspects of the plant under water restriction. However, new findings have shown that the development of *Arabidopsis* seeds *ABSCISIC ACID INSENSITIVE3* (*ABI3*), a transcription factor that contributes to the response to ABA, directly regulates miR156 genes in order to control maturation (Manuela & Xu, 2020; Tian et al., 2020). In addition, miR156/*SPL* has been linked to responses to stress, including hydrophilic stress, strengthening the relationship between ABA and miR156/*SPL* (Arshad et al., 2017; Feyissa et al., 2021).

ACC reflects endogenous ethylene levels, which control the regulation of leaf development and senescence, fruit maturation, apical hook formation, and biotic and abiotic stress responses (Dubois et al., 2018). The *STTM156* line showed high levels of ACC; this observation may be related to the age-dependent ethylene-induced senescence process (Li et al., 2013) since this genotype reaches adulthood early. Evidence of the interaction between ethylene and miR156/*SPL* has been described for the ripening process of papaya (*Carica papaya*), whose analysis of expression showed that the abundance of *SPLs* and miR156 was responsive to treatments with ethephon (ETH) and 1-methyl cyclopropane (1-MCP), precursor and ethylene inhibitor, respectively. miR156 has shown more abundance treatment with 1-MCP, a treatment in which *SPL2, SPL5* and *SPL9,* and SPL*4* transcripts were reduced (Xu et al., 2020).

In Passifloraceae, the presence of EFN is a common feature. In the leaves, they can be found on the leaf blade and petiole (Rocha et al., 2020; De Lemos et al., 2017). Here, it was possible to demonstrate that the module miR156-SPL affected the development of EFNs in *P. cincinnata*. The effects of miR156 were more drastic in EFNs present in leaf margins than in petiolar EFNs. In *P.*

cincinnata plants, marginal EFNs can be found from the juvenile stage when the leaves are trilobate; during the transition phase, the leaves gradually form deeper lobules until the appearance of the first pentalobed marking the beginning of the adult phase. Over these changes in leaf morphology, the number and size of EFNs in leaf margins increase (Fig.7); the number of petioles EFNs remains the same, and they show slight size variation during the transition between vegetative phases. Higher levels of miR156 have led to a decrease in the number of EFNs and affected size. In silenced lines, there was deregulation in leaf development, and the number of EFNs in the leaf margin was reduced. Unlike the NT plants, which presented larger nectaries in the pentalobed leaf, the STTM lines did not demonstrate a significant increase in EFN size after the transition of trilobed-to-pentalobed leaves, suggesting that the growth dynamics of EFNs were impacted at a certain level. These observations indicate that the miR156/SPL pathway participates in the regulation of the development of EFNs, simultaneously suggesting that the effect on nectary formation is indirect and that the lack of lobule formation prevents nectaries from forming.

miR156/*SPLs* is involved in regulating several biological processes, such as foliar heteroblasty, root development, and defense against pathogens (Rubio-Somoza et al., 2014, Silva et al., 2019, Mao et al.,2017, Barrera-Rojas et al., 2019), impacting at the transcriptional or protein level its targets. Leichty & Scott Poethig (2019) stated that the production of structures that promote indirect defense to *V. cornigera* and *V. collinsii* depends on age, and as proposed, like a new regulatory paper for miR156/*SPL*. As miR156/157 levels decreased, the length of EFNS and swollen stipular spines increased in *V. collinsii.* The pattern of SPL transcripts directed to miR156 was congruent with the development of structures that favor the mutualism between plants and ants to protect against herbivores. In *Passiflora organensis* and *P. edulis,* EFNs are found only in adult leaves (Moraes et al., 2022; Soares, 2020). The change in miR156/*SPL* levels influenced the structural organization of the tissues that make up the EFNs.

In general, EFNs are anatomically divided into the nectariferous epidermis, nectariferous parenchyma, and subnectariferous parenchyma, constituting the functional unit of this structure (Moraes et al., 2022; De Lemos et al., 2017). The *OE::miR156* line showed more drastic involvement of the tissues of the nectariferous parenchyma and EFN subnectariferous of the margin with a smaller set of cells composing the EFNs, culminating in the reduction of the size of the EFNs. In addition, the cells of the nectariferous parenchyma were more elongated. EFNs have anatomy and competent tissues for the production and exudation of nectar, and changes in tissues and size can lead to a decrease in nectar supply compared to cultivated plants that show a fine regulation of the development of EFNs. Buckwheat studies showed a strong relationship between nectar secretion and plant age and position in floral nectaries in inflorescence, where younger inflorescences had higher nectar production (Cawoy et al., 2008). Further studies are needed to understand the impact of the modulation of miR156/*SPL* levels on nectar production and exudation of EFNs in P. *cincinnata*.

The EFNs promote interaction with ants that act against herbivores, performing the function of defensive agents; in return, plants can offer shelter, sugar compounds, or secondary metabolites. The visitation of ants was lower in all transgenic genotypes, and in *OE::miR156* plants, this interaction was more compromised. The behavior of ants can be explained, at least in part, by the reduction in the number and size of EFNs in *OE::miR156* and *STTM156* plants. Other variables, such as secretion rate, secretion time, and nectar composition, may help explain the variation; however, other assays should be established to determine these parameters. Leal et al. (2006) identified a protective role of several ant species *in P. coccinea* against herbivores and nectar thieves, improving the reproductive success of plants. However, the effectiveness of this mutualism between plants and insects is limited. For example, although ants that promote mutualism with *P. caerulea* have a negative effect on the number of eggs and larvae of the Gulf fritillary butterfly (*Agraulis vanillae*), the level of herbivorous damage not decreased (Simonetti and Devoto, 2018). *Passiflora* species have EFNs with different structures, sizes, and locations (Lemos et al., 2017), and these characteristics influence the choice of ants; Comparing *P. auriculata*, *P. biflora*, and *P. oerstedii* was demonstrated that species with larger nectaries attracted more ants than species with smaller nectaries, more rapidly avoiding possible damage (Apple and Feener 2001).

Considering the increase in the number of nectaries along the vegetative phases in *P. cincinnata*, the recruitment of ants during adulthood is prioritized. Here, the visiting ants of *P. cincinnata* were identified as *Brachymyrmex* spp., an opportunistic in soil and vegetation (Fernández, 2003), which avoids aggressive interactions with other species and feeds from various sources, such as larvae and nectar (Cerpa et al., 2015). With the analyses performed, it is not possible to state if there is a mutualism relation. However, we show that *Brachymyrmex spp.* preference is age-dependent. Similarly, Moya-Raygoza and Larsen, (2014) showed that

Brachymyrmex obscurior feeds on the nectar of the *Acacia pennatula* but did not offer a protective benefit when visiting. It is shown that the interaction between the plant and ants via EFN is highly variable, and ants do not always benefit plants and can only benefit themselves from the resources that the plant offers; the ability to defend one another often depends on the species of ants that visit the nectaries (Koptur, 1984; Koptur and Lawton, 1988; Leal et al., 2006).

CONCLUSION

Regulation of leaf development requires a fine-tuning of miR156/*SPL* levels to define leaf characteristics at each development stage of development. In *P. cincinnata*, the increase in the number of lobes, which indicates phase transition, is related to the increase in nectaries numbers in the margin leaf. Thus, the miR156/*SPLs* module affects ecological interactions indirectly by regulating the growth and development of glandules such as extrafloral nectars, which are essential in competitive and mutualistic interactions. Our study provides new insights into the miR156/*SPL* pathway regulating leaf lobes formation and nectaries. Thus, *P. cincinnata* offers fewer rewards through the nectaries in the juvenile phase. In its adult phase, when the production phase is anticipated, a more significant investment of resources occurs in nectaries and exudates, facilitating the establishment of a mutualistic relationship with ants that can act as defenders.

REFERENCES

Apple, J.L. and Feener, D.H. (2001). Ant visitation of extrafloral nectaries of Passiflora: The effects of nectary attributes and ant behavior on patterns in facultative ant-plant mutualisms (Oecologia).

Arshad, M., Feyissa, B.A., Amyot, L., Aung, B., and Hannoufa, A. (2017). MicroRNA156 improves drought stress tolerance in alfalfa *(Medicago sativa)* by silencing SPL13. Plant Sci. **258**: 122–136.

Bowman, J.L. and Smyth, D.R. (1999). *CRABS CLAW*, a gene that regulates carpel and nectary development in Arabidopsis, encodes a novel protein with zinc finger and helix-loop-helix domains. Development **126**: 2387–2396.

Cerpa, P., Medrano, F., and Segura, B. (2015). Introducción a la ecología de las hormigas de la Región Metropolitana. La Chiricoca **19**.

Chitwood, D.H. and Otoni, W.C. (2017). Divergent leaf shapes among Passiflora species arise from a shared juvenile morphology. Plant Direct **1**: 1–15.

Curaba, J., Singh, M.B., and Bhalla, P.L. (2014). MiRNAs in the crosstalk between phytohormone signalling pathways. J. Exp. Bot. **65**: 1425–1438.

Dubois, M., Van den Broeck, L., and Inzé, D. (2018). The pivotal role of ethylene in plant growth. Trends Plant Sci. **23**: 311–323.

Eviatar-Ribak, T., Shalit-Kaneh, A., Chappell-Maor, L., Amsellem, Z., Eshed, Y., and Lifschitz, E. (2013). A cytokinin-activating enzyme promotes tuber formation in tomato. Curr. Biol. **23**: 1057–1064.

Feng, S., Xu, Y., Guo, C., Zheng, J., Zhou, B., Zhang, Y., Ding, Y., Zhang, L., Zhu, Z., Wang, H., and Wu, G. (2016). Modulation of miR156 to identify traits associated with vegetative phase change in tobacco (*Nicotiana tabacum*). J. Exp. Bot. **67**: 1493–1504.

Feyissa, B.A., Amyot, L., Nasrollahi, V., Papadopoulos, Y., Kohalmi, S.E., and Hannoufa, A. (2021). Involvement of the miR156/SPL module in flooding response in *Medicago sativa*. Sci. Rep. **11**: 1–16.

He, J., Xu, M., Willmann, M.R., McCormick, K., Hu, T., Yang, L., Starker, C.G., Voytas, D.F., Meyers, B.C., and Poethig, R.S. (2018). Threshold-dependent repression of *SPL* gene expression by miR156/miR157 controls vegetative phase change in *Arabidopsis thaliana*. PLoS Genet. **14**: e1007337.

Izaguirre, M.M., Mazza, C.A., Astigueta, M.S., Ciarla, A.M., and Ballaré, C.L. (2013). No time for candy: Passionfruit (Passiflora edulis) plants down-regulate damage-induced extrafloral nectar production in response to light signals of competition. Oecologia **173**: 213–221.

Koptur, S. (1984). Experimental evidence for defense of Inga (Mimosoideae) saplings by ants. Ecology **65**: 1787–1793.

Koptur, S. and Lawton, J.H. (1988). Interactions among vetches bearing extrafloral nectaries, their biotic protective agents, and herbivores. Ecology **69**: 278–283.

Kwok, K.E. and Laird, R.A. (2012). Plant age and the inducibility of extrafloral nectaries in *Vicia faba*. Plant Ecol. **213**: 1823–1832.

Lawrence, E.H., Leichty, A.R., Doody, E.E., Ma, C., Strauss, S.H., and Poethig, R.S. (2021a). Vegetative phase change in *Populus tremula* × *alba*. New Phytol. **231**: 351–364.

Lawrence, E.H., Springer, C.J., Helliker, B.R., and Poethig, R.S. (2021b). MicroRNA156-mediated changes in leaf composition lead to altered photosynthetic traits during vegetative phase change. New Phytol. **231**: 1008–1022.

Le, J. et al. (2014). Auxin transport and activity regulate stomatal patterning and development. Nat. Commun. 2014 51 **5**: 1–8.

Leal, I.R., Fischer, E., Kost, C., Tabarelli, M., and Wirth, R. (2006). Ant protection against herbivores and nectar thieves in *Passiflora coccinea* flowers. Ecoscience **13**: 431–438.

Leichty, A.R. and Scott Poethig, R. (2019). Development and evolution of age-dependent defenses in ant-acacias. Proc. Natl. Acad. Sci. U. S. A. **116**: 15596–15601.

De Lemos, R.C.C., Da Costa Silva, D., and De Albuquerque Melo-de-Pinna, G.F. (2017). A structural review of foliar glands in Passiflora L. (Passifloraceae). PLoS One **12**: e0187905.

Li, Z., Peng, J., Wen, X., and Guo, H. (2013). *ETHYLENE-INSENSITIVE3* is a senescence-associated gene that accelerates age-dependent leaf senescence by directly repressing miR164 transcription in Arabidopsis. Plant Cell **25**: 3311–3328.

Linh, N.M., Verna, C., and Scarpella, E. (2018). Coordination of cell polarity and the patterning of leaf vein networks. Curr. Opin. Plant Biol. **41**: 116–124.

Liu, Q., Zhang, Y.C., Wang, C.Y., Luo, Y.C., Huang, Q.J., Chen, S.Y., Zhou, H., Qu, L.H., and Chen, Y.Q. (2009). Expression analysis of phytohormone-regulated microRNAs in rice, implying their regulation roles in plant hormone signaling. FEBS Lett. **583**: 723–728.

Manuela, D. and Xu, M. (2020). Juvenile leaves or adult leaves: Determinants for vegetative phase change in flowering plants. Int. J. Mol. Sci. **21**: 1–16.

Moraes, T.S., Rossi, M.L., Martinelli, A.P., and Dornelas, M.C. (2022). Morphological and anatomical traits during development: Highlighting extrafloral nectaries in *Passiflora organensis*. Microsc. Res. Tech. **85**: 2784–2794.

Moya-Raygoza, G. and Larsen, K.J. (2014). Response of ants to the leafhopper *Dalbulus quinquenotatus* DeLong & Nault (Hemiptera: Cicadellidae) and extrafloral nectaries following fire. Sociobiology **61**: 136–144.

Napoleão, T.A., Soares, G., Vital, C.E., Bastos, C., Castro, R., Loureiro, M.E., and Giordano, A. (2017). Methyl jasmonate and salicylic acid are able to modify cell wall but only salicylic acid alters biomass digestibility in the model grass *Brachypodium distachyon*. Plant Sci. **263**: 46–54.

Nguyen, S.T.T., Greaves, T., and McCurdy, D.W. (2017). Heteroblastic development of transfer cells is controlled by the microRNA *miR156/SPL* module. Plant Physiol. **173**: 1676–1691.

Nicolson, S.W., Nepi, M., and Pacini, E. (2007). Nectaries and nectar (Springer Netherlands).

Pinheiro, F. (2007). Caracterização da variabilidade morfoagrônomica de maracujazeiro (*Passiflora cincinnata* Mast.) no semi-árido.

Rocha, D.I. et al. (2020). Passiflora spp. Passionfruit. Biotechnol. fruit nut Crop.: 381–408.

Roy, R., Schmitt, A.J., Thomas, J.B., and Carter, C.J. (2017). Review: Nectar biology: From molecules to ecosystems. Plant Sci. **262**: 148–164.

Rubio-Somoza, I., Zhou, C., Confraria, A., Martinho, C., von Born, P., Baena-Gonzalez, E., Wang, J., and Weigel, D. (2014). Temporal control of leaf complexity by miRNA-regulated licensing of protein complexes. Curr. Biol. **24**: 2714–2719.

Shwartz, I., Levy, M., Ori, N., and Bar, M. (2016). Hormones in tomato leaf development. Dev. Biol. **419**: 132–142.

Silva, E.O., Feio, A.C., Cardoso-Gustavson, P., Milward-De-Azevedo, M.A., Dos Santos, J.U.M., and De Aguiar Dias, A.C.A. (2017). Extrafloral nectaries and plant–insect interactions in Passiflora L. (Passifloraceae). Rev. Bras. Bot. **40**: 331–340.

Silva, G.F.F.E., Silva, E.M., Da Silva Azevedo, M., Guivin, M.A.C., Ramiro, D.A., Figueiredo, C.R., Carrer, H., Peres, L.E.P., and Nogueira, F.T.S. (2014). MicroRNA156-targeted SPL/SBP box transcription factors regulate tomato ovary and fruit development. Plant J. **78**: 604–618.

Silva, P.O., Batista, Di.S., Cavalcanti, J.H.F., Koehler, A.D., Vieira, L.M., Fernandes, A.M., Barrera-Rojas, C.H., Ribeiro, Di.M., Nogueira, F.T.S., and Otoni, W.C. (2019). Leaf heteroblasty in *Passiflora edulis* as revealed by metabolic profiling and expression analyses of the microRNAs miR156 and miR172. Ann. Bot. **123**: 1191–1203.

Simonetti, G. and Devoto, M. (2018). La defensa de *Passiflora caerulea* por hormigas reduce el número de huevos y larvas de Agraulis vanillae, pero no el daño por herbivoría. Ecol. Austral **28**: 123–132.

Soares, R.J. (2020). Efeitos da superexpressão do microRNA156 sobre a morfofisiologia de *Passiflora edulis* Sims. durante a transição de fase juvenil/ adulta.

Toriba, T., Tokunaga, H., Shiga, T., Nie, F., Naramoto, S., Honda, E., Tanaka, K., Taji, T., Itoh, J.I., and Kyozuka, J. (2019). *BLADE-ON-PETIOLE* genes temporally and developmentally regulate the sheath to blade ratio of rice leaves. Nat. Commun. **10**: 1–13.

Wang, J.W., Park, M.Y., Wang, L.J., Koo, Y., Chen, X.Y., Weigel, D., and Poethig, R.S. (2011). MiRNA control of vegetative phase change in trees. PLoS Genet. **7**: e1002012.

Werner, S., Bartrina, I., and Schmülling, T. (2021). Cytokinin regulates vegetative phase change in *Arabidopsis thaliana* through the miR172/TOE1-TOE2 module. Nat. Commun. **12**: 1–11.

Xu, Y., Xu, H., Wall, M.M., and Yang, J. (2020). Roles of transcription factor *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* gene family in papaya (*Carica papaya*) development and ripening. Genomics **112**: 2734–2747.

Yu, S., Lian, H., and Wang, J.W. (2015). Plant developmental transitions: The role of microRNAs and sugars. Curr. Opin. Plant Biol. **27**: 1–7.

Zhang, T.Q., Lian, H., Tang, H., Dolezal, K., Zhou, C.M., Yu, S., Chen, J.H., Chen, Q., Liu,

H., Ljung, K., and Wang, J.W. (2015). An intrinsic microRNA timer regulates progressive decline in shoot regenerative capacity in plants. Plant Cell **27**: 349–360.

GENERAL CONCLUSION

Parameters widely described in the literature were validated. The *OE::miR156* line presented lower height, reduced phytomer size, reduced growth, and correlative inhibition, contrasting with NT and the silenced lines. However, the silenced lines were similar to NT, possibly due to a reduction in miR156 free levels, which stopped inhibiting all its target SPLs simultaneously and continuously. Creating constructs to silence SPLs individually will allow an understanding of the role of each in the growth and development of *P. cincinnata*.

The differences in tendril development suggest that the miR156/SPLs module does not play a central role in integrating age signals to promote the first tendril appearance. Still, it affects the growth and development of the tendrils and directly affects the successful support and climbing of the plant. It is essential to determine if there are differences in the development of the tendrils in growth conditions that simulate the natural environment to define how miR156 regulates their development and, besides, figure out what determines the appearance of the first tendril.

We found that the miR156/SPLs module is essential to define leaf characteristics at each development stage and, therefore, lobules formation during the phase transition in *P. cincinnata*. However, the increase in the lobes numbers is closely related to the increase in nectaries numbers in the margin leaf. Thus, this module indirectly affects ecological interactions, regulating the growth and development of glandules such as extrafloral nectars, essential in competitive and mutualistic interactions. Our study provides new insights into the miR156/SPL pathway regulating leaf lobes formation and nectaries.