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Beyond leaf development: roles of the miR319/*LANCEOLATE* module
in controlling tomato meristem size and flowering

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Thesis presented to obtain the degree of Doctor in
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RESUMO

Além do desenvolvimento foliar: papéis do módulo miR319/*LANCEOLATE* no controle do tamanho do meristema e florescimento de tomateiro

O florescimento é uma das etapas de desenvolvimento mais importantes no ciclo de vida da planta. O tempo de florescimento adequado, e o correto desenvolvimento das inflorescências e flores são determinantes para o sucesso reprodutivo e a produtividade de culturas vegetais. Muitas características do florescimento são controladas por processos que ocorrem no meristema apical da parte aérea, já na fase vegetativa. Processos que vão desde o estabelecimento do tamanho do meristema vegetativo, sua maturação, até os últimos estágios do desenvolvimento dos verticilos florais, determinam as características reprodutivas de uma planta. Fatores de transcrição regulados por microRNAs (miRNAs) desempenham papéis cruciais na maioria dessas etapas. Os miRNAs são responsáveis pela regulação quantitativa e espaço-temporal de seus alvos, fornecendo padrões de expressão ideais para o desenvolvimento de órgãos. Muitas vias reguladas por miRNAs têm sido descritas como essenciais na regulação do desenvolvimento foliar, transição juvenil para adulto, tempo de florescimento e amadurecimento dos frutos, entre outros estágios de desenvolvimento. Fatores de transcrição do tipo *TEOSINTE BRANCHED1/ CYCLOIDEA/ PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR1* (*TCPs*), vários dos quais são regulados pelo miR319 (denominados *CINCINNATA-like TCPs* ou *CIN-TCPs*), tem um papel bem descrito e conservado no desenvolvimento foliar. Dentre os *CIN-TCPs*, o papel do módulo miR319/*LANCEOLATE* (*LA*) no florescimento parece divergir entre *Arabidopsis* e tomateiro. Além disso, o mecanismo pelo qual o módulo miR319/*LA* controla o florescimento do tomateiro não é totalmente compreendido. Considerando isso, aqui nós: (1) revisamos a literatura disponível sobre os principais conceitos de maturação do meristema, transição para o florescimento, desenvolvimento da inflorescência e desenvolvimento floral e (2) estudamos como o *hub* miR319/*CIN-TCP LA* se interconecta com diferentes vias na regulação do tamanho do meristema e florescimento. Nós mostramos que a regulação fina fornecida por este *hub* controla o tamanho do meristema vegetativo, a transição para o florescimento e o desenvolvimento da flor. O módulo miR319/*LA* atua regulando o ciclo celular e interagindo com múltiplas vias de fitohormônios, culminando em um ajuste fino do desenvolvimento reprodutivo de tomateiro.

Palavras-chave: miR319, TCPs, *LANCEOLATE*, Giberelina, *DELLA*, Florescimento, Tamanho de meristema

ABSTRACT

Beyond leaf development: roles of the miR319/*LANCEOLATE* module in controlling tomato meristem size and flowering

Flowering is one of the most important developmental stages in the plant life cycle. The proper flowering time, and the correct development of inflorescences and flowers are crucial for the reproductive success and vegetable crops yield. Many flowering characteristics are controlled by processes that occur in the shoot apical meristem, already in the vegetative phase. Processes ranging from the establishment of vegetative meristem size, its maturation, to the last stages of the development of floral whorls, determine the reproductive characteristics of a plant. MicroRNA-regulated transcription factors (miRNAs) play crucial roles in most of these steps. MiRNAs are responsible for the quantitative and spatiotemporal regulation of their targets, providing ideal expression patterns for organ development. Many pathways regulated by miRNAs have been described as essential in the regulation of leaf development, juvenile to adult transition, flowering time, and fruit ripening, among other developmental stages. *TEOSINTE BRANCHED1/ CYCLOIDEA/ PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR1* transcription factors (TCPs), several of which are regulated by miR319 (called *CINCINNATA-like TCPs* or *CIN-TCPs*), have a well-described and conserved role in leaf development. Among the *CIN-TCPs*, the role of the miR319/*LANCEOLATE* (*LA*) module in flowering seems to differ between *Arabidopsis* and tomato. Furthermore, the mechanism by which the miR319/*LA* module controls tomato flowering is not fully understood. Taking that into account, here we: (1) review the available literature on the main concepts of meristem maturation, transition to flowering, inflorescence development, and floral development, and (2) study how the miR319/*CIN-TCP LA* hub interconnects with different pathways in the regulation of meristem size and flowering. We show that the fine regulation provided by this hub controls vegetative meristem size, transition to flowering and flower development. The miR319/*LA* module acts by regulating the cell cycle and interacting with multiple phytohormone pathways, culminating in a fine-tuning of tomato reproductive development.

Keywords: miR319, TCPs, *LANCEOLATE*, Gibberellin, *DELLA*, Flowering, Meristem size

1. TEOSINTE BRANCHED1/ CYCLOIDEA/ PCN (TCP) TRANSCRIPTION FACTORS, GIBBERELLIN (GA), REGULATION OF MERISTEM SIZE AND MATURATION, AND FLOWERING

Abstract

Plants are very plastic organisms that perceive and respond very quickly to environmental changes in order to adapt and survive. The meristems, a niche of stem cells, are the main responsible for this plasticity, being able to differentiate into new organs. The final inflorescence architecture and development depends on the activity of the shoot apical meristem (SAM). After plant germination, SAM initiate the production of leaves and start a maturation process. Here we reviewed main concepts of meristem size, maturation and transition, inflorescence, and flower development, including the role of transcriptions factors, microRNAs (miRNAs) and phytohormones in these processes, with a special focus on the role of miR319-regulated *TEOSINTE BRANCHED1/ CYCLOIDEA/ PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR1 (TCPs)*, which seem to have a relevant role in all these steps.

Keywords: meristem size, flowering, flower development, *DELLA,* miR319/*LANCEOLATE*

1.1. Introduction

Different from the animals, plants are sessile organisms, thus they must be plastic and quickly modulate their shoot architecture in response to endogenous cues to adapt to the environment and thrive. Shoot architecture is one of the main developmental factors affecting crop productivity and it is influenced by both vegetative and reproductive structures. The body of the plant is established by meristems, which contain a pool of stem cells continuously providing new cells to form new tissues (Holt et al., 2014). In the shoot apex, the shoot apical meristem (SAM) contains smalls groups of pluripotent cells that give rise to new lateral organs, such as leaves and flowers. One key change in SAM identity is the transition to flowering or meristem maturation, in which the SAM transitions to inflorescence meristem (IM) and floral meristem (FM) (Benlloch et al., 2007; Kwiatkowska, 2008). Therefore, the final inflorescence architecture and shoot shape depend on the formation of the meristem and on the transition to the reproductive phase. This transition is tightly associated with temporal and spatial changes of growth at the SAM (Kwiatkowska, 2008). Both meristem size and meristem transitions depend on different factors, including phytohormone- and microRNA-regulated genetic pathways. In the next sections, we will discuss each factor separately and their possible interconnection to control flowering.

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2. THE miR319/*LANCEOLATE* HUB INTERCONNECTS PHYTOHORMONES, CELL CYCLE REGULATION AND THE FLOWER ACTIVATING COMPLEX TO CONTROL TOMATO FLOWERING

Abstract

Flowering is a key developmental transition from vegetative to reproductive growth. For crops, the appropriated flowering time and flower development are closely related to yield. During the flowering transition, the shoot apical meristem (SAM) stops producing leaves and stems and start to produce flowers. Therefore, changes that take place very early, still in the vegetative meristem, can determine the flowering time, inflorescence architecture and the number of flowers. Most knowledge regarding flowering is based on the day-length responsive *Arabidopsis*. Conversely, little is known about flowering in the day-neutral tomato. We have previously shown that the miR319-targeted *CIN LANCEOLATE* controls flowering time by interacting with gibberellin signaling pathway through the DELLA/PROCERA protein. Nevertheless, the molecular mechanisms by which miR319-tageted *CIN-TCPs* modulate meristem activity and flowering in tomato remain largely unknown. Here, we combined genetic and molecular tools to characterize in detail how the miR319/*CIN-TCP* hub modulates meristem size and flowering in tomato. Our data are consistent with miR319/*LA* directly controlling the expression of cell cycle related genes in vegetative SAM and controlling GA homeostasis and ethylene (ETH) pathway, thus impacting GA- and ETH controlled flowering. Moreover, tomato miR319-targeted TCPs regulate flowering by protein interaction with DELLA/PROCERA and with the main genes of the Flowering Activation Complex (FAC). Our results provide a new mechanism by which the miR319/*TCPs* hub regulates meristem area and flowering, and suggest that LANCEOLATE and their targets have potential applications in improving tomato reproductive development.

Keywords: miR319; TCPs; *LANCEOLATE*; *DELLA*, flowering, meristem size

2.1 Introduction

The onset of flowering is one of the most important transitions in plant development, determining the reproductive success of a specie. In tomato (*Solanum lycopersicum*), an important vegetable crop, flowering is also a crucial feature related to yield. The shoot apical meristem (SAM), a group of undifferentiated cells, give rise to all shoot organs, such as stem and leaves. An integration of internal and external cues induces SAM to produce flower-bearing shoots or differentiate direct into flowers (Benlloch et al., 2007; Kwiatkowska, 2008).

Therefore, the final shoot architecture depends on how the meristems transition to reproductive phase. This transition is called meristem maturation and it is regulated at the genetic and epigenetic levels by an intricate regulatory network, which include phytohormones, transcriptional regulation and small non-coding regulatory RNAs (Silva et al., 2019; Park et al., 2012). In the model plant *Arabidopsis thaliana*, meristem maturation is regulated by the integration of five pathways, including vernalization, autonomous, gibberellin, age and photoperiod (Fornara et al., 2010; Andrés and Coupland, 2012). These pathways usually converge on the regulation of the flowering integrators, *FLOWERING LOCUS T (FT)* and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* ((Kardailsky et al., 1999; Kobayashi et al., 1999; Lee et al., 2000).

MicroRNAs and targets are promising candidates as fine-tuners for complex traits such as shoot architecture and flowering (Tan and Chu, 2017). The miR156 and its targets - members of the transcription factor family SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL or SBP-box)- have been proposed to influence several traits, including the timing of vegetative and reproductive phase change (Wang and Wang, 2015; Wei et al., 2018). In contrast to the miR156-*SPL/SBP* module, there are few reports describing the role of miR319/*TCP* module in regulating floral induction or repression. The miR319 targets a sub-group of the *TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP)* family of TFs, called *CINCINNATA-like TCPs* or *CIN-TCPs*. In both tomato and *Arabidopsis,* the *CIN-TCP* repression by miR319 is fundamental to proper leaf development (Palatnik et al., 2003; Ori et al., 2007). In tomato, the *CIN-TCP LANCEOLATE (LA, AtTCP4* homolog) promotes leaf maturation and the de-repression of *LA* leads to simpler leaves in the semi-dominant *Lanceolate (La)* mutant. (Ori et al., 2007; Shleizer-Burko et al., 2011).

In *A. thaliana*, the over-accumulation of miR319 leads to a late flowering phenotype, while plants expressing miR319-resistant versions of *TCPs* flower earlier (Li et al., 2019). In contrast, tomato plants expressing miR319 under the control of the constitutive *FILAMENTOUS FLOWER (FIL)* promoter flower earlier, while the semi-dominant *La* mutant presented a late flowering phenotype (Burko et al., 2013; Silva et al., 2019). Moreover, it has been shown that miR319-targeted TCPs positively regulate the levels of bioactive GAs in both *Arabidopsis* and tomato, which might contribute to the TCPs role in modulating flowering time and shoot architecture (Liu et al., 2017; Burko et al., 2013; Silva et al., 2019). Nevertheless, the molecular mechanisms by which miR319-tageted *CIN-TCPs* modulate flowering in tomato remain largely unknown.

The phytohormone GA is well known as regulator of reproductive development (Evans and Poethig, 1995). GA regulates developmental pathways mostly through nuclear DELLA proteins, a negative regulator of GA signaling (Hauvermale et al., 2012; Porri et al., 2012). In *Arabidopsis*, the accumulation of DELLA proteins delays flowering independent of the photoperiod (Hauvermale et al., 2012). DELLAs can repress flowering by acting in both leaves and in the SAM (Galvão et al., 2012; Porri et al., 2012). *Arabidopsis* DELLAs can also restrict the inflorescence meristem size by activating cell cycle inhibitors genes like *KIP-related proteins* (Serrano-Mislata et al., 2017). Changes in the meristem size can largely affect reproductive development, modifying the number of floral buds formed (inflorescence architecture) and the phyllotaxis (Leyser and Furner, 1992). More recently, the floral transition-associated regulatory module (miR172/*APETALA2* or *AP2*) was shown to also control inflorescence meristem size, thus fine-tuning *Arabidopsis* SAM determination and maturation (Sang et al., 2022).

Interesting, PROCERA (PRO) (tomato DELLA; Bassel et al., 2008) degradation by increased GA levels delays flowering in tomato (Silva et al., 2019). In leaves, exogenous GA application and *pro* mutation suppresses the indeterminate growth phenotype in miR319 overexpression plants, indicating that GA can influence the LA activity (Yanai et al., 2011). Furthermore, miR319-targeted TCP19 interacts with the DELLA protein REPRESSOR OF ga1-3 (RGA) and synergistically inhibited the induction of *GLABRA2* (*GL2*) gene, thereby repressing trichome initiation in *Populus tomentosa* leaves (Fan et al., 2020). We have preciously shown that LANCEOLATE can interact with PROCERA/DELLA (Silva et al., 2019). This interaction suggests a mechanistic relationship between TCPs and GA pathways in the regulation of flowering and meristem size in tomato.

Ethylene (ETH) is another phytohormone that can influence flowering. The stress-related phytohormone ethylene is well known as growth modulator in response to environmental stimuli (Achard et al., 2006). ETH is perceived by the ETR family of membrane receptors. In the absence of ethylene, the receptor directly interacts and activates CTR1, a negative regulator of downstream events (Kieber et al., 1993; Huang et al., 2003). Several positive regulators of ethylene signaling are downstream CTR1, such as EIL family of transcriptions factors members EIN2 and the EIN3, and EIN3-like (EIL). EIN3 is responsible for the activation of ethylene responsive genes (Chao et al., 1997; Solano et al., 1998). In *Arabidopsis*, ethylene delays flowering by reducing GA levels and enhancing the accumulation of DELLA proteins, via CTR1-dependent pathway (Achard et al., 2006). However, the effect of ethylene in tomato flowering remains largely unknown.

Here we show that miR319-dependent regulation of *LANCEOLATE* in tomato apices is fundamental to modulate meristem size, flowering transition, and inflorescence architecture. De-repression of *LA* in tomato vegetative apices restrict the meristem size and increases the time for meristem transition. The opposite was observed for plants accumulating high levels of miR319. Our genetic and molecular data are consistent with LA directly repressing cell cycle inhibitor genes such as tomato *SlKRP2* and *SlKRP7* in vegetative SAM, and acting synergistically with DELLA in the meristem size restriction. Our results also indicate that miR319-targeted *LA* controls GA homeostasis in vegetative apices by repressing GA degradation genes like *SlGA2ox2* and *SlGA2ox5*, and controls ethylene signaling pathway, thus impacting GA- and ETH-controlled flowering. We also show that *LA* does not affect the expression of flowering integrators genes at the SAM, but rather it interacts with SINGLE FLOWER TRUSS (SFT) and SELF-PRUNING G-BOX PROTEIN (SPGB) at the protein level. Tomato plants with high levels of LA and SFT in the shoot apices exhibited simpler inflorescences, suggesting that the meristem maturation was accelerated in such plants. Taken together, these results define a miRNA-controlled hub that interconnects phytohormones, cell cycle regulation and flowering integrators to control meristem maturation and flowering in tomato.

2.2 Conclusions

The data presented in this Thesis provide new evidence of the role of miR319/*TCPs* module in controlling different aspects of tomato development, as meristem size, flowering transition, inflorescence and flower development. In general lines, it is possible to conclude that:

1. The spatio-temporal regulation of the CIN-TCP *LANCEOLATE* by miR319 in the tomato vegetative phase controls meristem size, as LA directly activates cell cycle-associated genes. Furthermore, LA interplays with DELLA/PRO either antagonistically in the control of flowering transition or synergistically in the restriction of meristem size.

2. LA physically interacts with the two main proteins of the Flowering Activation Complex (FAC), and interferes with the flowering induction activity of the SFT protein.

Working with a non-model plant can be very challenging. During this work many protocols and pipelines had to be adapted from other species, mainly from the model plant *Arabidopsis*. Other important consideration is regarding the necessity of a tissue-culture based transformation protocol for generating tomato transgenic plants, which, in addition to the longer tomato life cycle, makes it more difficult to quickly create tools for more genetic and molecular confirmations, mainly for the non-dwarf M82 cultivar. In addition, even though an enormous progress has been made in recent years, tomato genome assembly is still lacking a considerable number of gene functional annotations, which is a challenge in sequencing analyses. Despite of the challenges, this work expands the knowledge on how pathways controlled by miRNAs can significantly affect development and shed some light to an important question in plant developmental biology: how plants regulate meristem size and how this regulation impacts reproductive development, which is fundamental for crop yield.

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