University of São Paulo
"Luiz de Queiroz" College of Agriculture

Tomato gynoecium patterning and fruit development are orchestrated by the interplay between microRNAs and gibberellin

João Paulo de Oliveira Corrêa

Thesis presented to obtain the degree of Doctor in Science. Area: Plant Physiology and Biochemistry

Piracicaba
2019

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Bachelor in Biological Sciences

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Corrêa, João Paulo de Oliveira

Tomato gynoecium patterning and fruit development are orchestrated by the interplay between microRNAs and gibberellinl / João Paulo de Oliveira Corrêa. - - Piracicaba, 2019.

80 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Tomateiro 2. MiR156 3. Giberelina 4. Fruto 5. Gineceu I. Título

ACKNOWLEDGEMENTS

I would like to acknowledge everyone that, directly and indirectly, contributed to my thesis. I am deeply thankful to you all. Special thanks to:

My supervisor Dr Fabio Nogueira for being a very good supervisor, always supportive, involved in my work and available for fruitful scientific discussions. Thank you very much for the patience and knowledge shared.

My supervisor in John Innes Centre, Dr Robert Sablowski. Thank you for hosting me in your lab, supporting my research and sharing all the amazing knowledge on meristem development. I learned so many things in this visit that those six months looked like a much longer period to me.

Dr Lázaro Peres for the scientific knowledge shared, seed greenhouse structure and lab equipment provided during my PhD.

All my lab colleagues and friends from GMDV Antoine, Leticia, Diego, Carol, Mateus, Carlos and Felipe.

All colleagues and friends from LCHDV.

Geraldo Silva and Eder Silva for being practically my co-supervisors during my first PhD years and for the friendship we developed along the time. Thank you for the friendship, for teaching me from scratch how to work with molecular biology, for all the scientific discussion we always had.

Airton Carvalho and Marcela Notini for the friendship, help and for also being the guys who enjoy having a beer to talk about science.

My friends from John Innes Centre Marco, Emma, Max, Aura, Anna, Marina and Jesus. Thank you very much for the support, the knowledge shared and for making my adaptation to UK much easier. I am really thankful for meeting you all.

My friends Lorena, Andressa, Lucas and Italo for being my family in Piracicaba. For all the unforgettable moments we had together, in good times and bad times.

Danilo, for being always by my side giving me emotional support during the whole PhD time. Thank you very much for everything!

My parents Batista and Magda for absolutely everything. For all the love, for being examples of human beings to me.

My sister Marina for all the love and patience.

CNPq (process 140513/2015-9) and FAPESP (processes 2016/05073-4 and 2018/13316-0) for the financial support.

CONTENTS

RESUMO

O desenvolvimento do gineceu e fruto em tomateiro é coordenado pela interação entre giberelina e microRNAs

Muitas características finais deu um fruto são controladas por processos que se dão muito cedo no desenvolvimento dos frutos, até mesmo a nível de meristema. De fato, processos que vão desde a transição de um meristema vegetativo para um meristema de inflorescência até os estágios finais do amadurecimento definem muitas características do fruto maduro. Fatores de transcrição regulados pós transcricionalmente por microRNAs (miRNAs) têm papel crucial na maior parte desses estágios. Nessas vias os miRNAs geralmente regulam a expressão de seus alvos espacialmente, temporalmente, ou diminuem o acúmulo de transcritos do alvo, proporcionando níveis adequados de expressão para o desenvolvimento do órgão. Muitos estudos mostram que determinados miRNAs são essenciais no controle do tempo de florescimento, desenvolvimento do gineceu, crescimento do fruto após a polinização e amadurecimento, dentre outros estágios do desenvolvimento do fruto. Muitos aspectos do controle do desenvolvimento inicial do gineceu foram relativamente bem estudados em Arabidopsis. Porém, essas etapas iniciais do desenvolvimento de carpelos/gineceu ainda não são bem estudadas em plantas que produzem fruto carnoso, como é o caso do tomateiro. Já foi demonstrado que muitas vias reguladas por miRNAs interagem com fitormônios na regulação de vários processos de desenvolvimento. Como exemplo podemos citar a relação entre a via do miR156 e a via das giberelinas (GA) no controle do tempo de florescimento. Essa relação é substancialmente em Arabidopsis e tomateiro. Além disso, foi demonstrado que o miR156 possui papéis distintos na regulação do desenvolvimento do gineceu nessas duas espécies. Dessa forma, nesse trabalho nós: (1) fazemos uma revisão de literatura sobre a regulação do desenvolvimento de frutos, do desenvolvimento inicial do gineceu ao amadurecimento, por vias reguladas por RNAs não codantes e; (2) estudamos a relação entre as vias do miR156 e GA na regulação de etapas iniciais do desenvolvimento de frutos. Nós demonstramos que essas vivas controlam o tamanho de meristemas florais, bem como o estabelecimento de zonas de divisão no desenvolvimento do gineceu. Além disso, mostramos que a via do miR156 modula a resposta a GA.

Palavras-chave: Tomateiro; MiR156; Giberelina; Fruto; Gineceu

ABSTRACT

Tomato gynoecium patterning and fruit development are orchestrated by the interplay between microRNAs and gibberellin

Many characteristics of a fully developed fruit are controlled by processes that take place early in fruit development, even at floral meristem level. In fact, events that range from the transition of a vegetative into an inflorescence meristem to the last stages of fruit ripening determine characteristics of a mature fruit. Transcription factors post-transcriptionally regulated by microRNAs (miRNAs) play crucial roles in most of these stages. In such pathways, miRNAs may regulate its targets spatially, temporally, or dampen amount of targets transcript to provide optimal expression patterns for adequate organ development. Many miRNAs have been described to be essential in the regulation of flowering time, gynoecium patterning, post-pollination gynoecium growth and fruit ripening, among other stages of fruit development. Some aspects of early stages of gynoecium development, such as floral meristem maintenance, carpel fusion and gynoecium pattern, have been well studied in Arabidopsis. However, these stages are poorly understood in the development of fleshy fruit species, such as tomato. Many miRNA-related pathways were described to interact with phytohormone pathways. A good example is the interaction between miR156 and Gibberellins (GA) in the regulation of flowering time. Interestingly, this interaction is substantially different in Arabidopsis and tomato. MiR156 have been also shown to have distinct roles in controlling tomato and Arabidopsis gynoecium development. Considering this, here we: (1) review the available literature concerning control of fruit development, from gynoecium patterning to fruit ripening, by non-coding RNAs and (2) study the relationship between miR156 and GA pathways in the regulation of early stages of fruit development. We show that these pathways control floral meristem size and boundary establishment during gynoecium development and miR156 pathway modulates responses to GA.

Keywords: Tomato; MiR156; Gibberellin; Fruit; Gynoecia

1. MOLECULAR CONTROL BY NON-CODING RNAS DURING FRUIT DEVELOPMENT: FROM GYNOECIUM PATTERNING TO FRUIT RIPENING

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Running title: Non-coding RNAs in fruit development and ripening

Invited review article published in Frontiers in Plant Science

Abstract

Fruits are originated from the transition of a quiescent ovary to a fast-growing young fruit. The evolution of reproductive structures such as ovary and fruit has made seed dispersal easier, which is a key process for reproductive success in flowering plants. The complete fruit development and ripening are characterized by a remarkable phenotypic plasticity which is orchestrated by a myriad of genetic factors. In this context, transcriptional regulation by non-coding small (*i.e.*, microRNAs) and long (lncRNAs) RNAs underlie important mechanisms controlling reproductive organ development. These mechanisms may act together and interact with other pathways (*i.e.* phytohormones) to regulate cell fate and coordinate reproductive organ development. Functional genomics has shown that non-coding RNAs regulate a diversity of developmental reproductive stages, from carpel formation and ovary development to the softening of the ripped fruit. This layer of transcriptional control has been associated with ovule, seed, and fruit development as well as fruit ripening, which are crucial developmental processes in breeding programs because of their relevance for crop production. The final ripe fruit is the

result of a process under multiple levels of regulation, including mechanisms orchestrated by microRNAs and lncRNAs. Most of the studies we discussed involve work on tomato and *Arabidopsis*. In this review, we summarized non-coding RNA-controlled mechanisms described in the current literature that act coordinating the main steps of gynoecium development/patterning and fruit ripening.

Keywords: Tomato; Fruit development; MicroRNAs; IncRNAs; Ripening

1.1. Introduction

Fruits are plant organs found solely in angiosperms and are commonly defined as mature ovaries containing seeds. They are also ecologically defined as seed dispersal units, and their diversification and specialization are key events of the adaptive success of angiosperms in a wide range of environments (Seymour et al., 2013). The final characteristics of a mature fruit are determined by events that take place in developmental stages ranging from floral meristem initiation to later stages of fruit ripening. Complex mechanisms of transcriptional regulation of each of these stages ensure proper fruit development. After floral meristem initiation, key events of fruit development include carpel formation, differentiation, patterning and organ boundary formation. Ovule and seed development are also fundamental processes for the completion of fruit maturation. Fruit set occurs when the signaling triggered by the pollination and fertilization turns a fully developed ovary into a fast-growing fruit that will soon initiate the ripening process.

Although physiological and molecular aspects of fruit development and ripening are well discussed in the available literature (Ferrándiz et al., 2010; Liu et al., 2015), few reviews focused on the role of non-coding RNA-based molecular regulation controlling early and late stages of fruit development. Here, we reviewed the literature focused on the aspects of the regulation by non-coding RNA in different stages of fruit development, including ovule and seed development. Moreover, we discussed aspects of fruit growth and ripening in the light of miRNA and lncRNA-associated mechanisms. One important question that need to be better addressed in future studies is how transcriptional control of fruit development is conserved between dry fruit-bearing and fleshy fruit-bearing species (e.g, *Arabidopsis thaliana* and tomato or *Solanum lycopersicum*, respectively). A better understanding of the non-coding RNA-related transcription hallmarks orchestrating early steps of fruit development and ripening in different species may have the potential to provide novel strategies for crop improvement.

1.2. microRNA modules involved in early steps of fruit patterning and growth

The carpel is the female reproductive organ that encloses the ovules in flowering plants. The gynoecium is the innermost floral whorl, formed by the fusion of carpels in the center of the flower. The hypothesis of the origin of the carpels as modified leaves is corroborated by the observation that leaf development-associated factors also have roles in carpel development (Dinneny et al., 2005; Scutt et al., 2006; Alonso-Cantabrana et al., 2007; Ferrándiz et al., 2010; Gonzales-Reig et al., 2012; Seymour et al., 2013; Deb et al., 2018). Carpel and fruit development can be broadly divided into two main temporal set of events: an earlier set of events that occur prior to fertilization (differentiation and patterning), and later events, which occur after fertilization (growth, ripening and senescence) (Ripoll et al., 2015; Deb et al., 2018). A fine-tuned molecular regulation of each of these developmental steps is crucial to ensure proper morphological and physiological characteristics of the mature fruit.

MicroRNAs (miRNAs) and their targets (mostly transcription factors; Chen, 2009) are fundamental components of molecular modules (hereafter referred to microRNA modules) belonging to complex circuits that control various aspects of plant development. miRNAs inhibit the activity of their targets by two major mechanisms: *ARGONAUTE1 (AGO1)*-mediated transcriptional cleavage, and translational repression of gene targets (Borges and Martienssen, 2015). At cell and tissue levels, many miRNAs accumulate in a spatiotemporal manner to modulate and/or fine-tune the expression of their targets (Chen, 2009; Rubio-Somoza and Weigel, 2011). For instance, some miRNAs participate in tissue patterning by restricting the expression domain of target genes (Berger et al., 2009; Chen, 2009; Ripoll et al., 2015). On the other hand, miRNAs and targets may be co-expressed in similar domains, where miRNAs ensure proper transcript accumulation by dampening target transcript levels. In this case, miRNAs generally mediate the temporal control of transcript accumulation, in which cells and/or tissues exhibit a gradual decrease or increase in the levels of target transcripts as the organ develops (Wu et al., 2010; Rubio-Somoza et al., 2014; Wang, 2014; Guo et al., 2017; He et al., 2018).

Some miRNA modules had their roles in gynoecium and fruit development described in different model plants, such as *Arabidopsis thaliana*, which produces dry fruits (silique), and tomato (*Solanum lycopersicum*), which produces fleshy fruit (berry). Interestingly, alterations in similar miRNA modules produce distinct phenotypic changes in gynoecium and fruits of *Arabidopsis* and tomato (Xing et al., 2013; Silva et al., 2014). Understanding what pathways are directly and/or indirectly regulated by similar miRNA modules in different species, and how they influence distinct fruit morphologies, will shed light on important evolutionary aspects of fruit

development. In the next sections, we discussed examples in the literature concerning the roles of miRNA modules in early events of fruit development mostly in tomato and *Arabidopsis*.

1.2.1. The miR164 module controls carpel development and leaf margin serration through similar mechanisms

MiRNA-associated pathways control many aspects of plant development. Some miRNA-targeted transcriptional regulators that had their roles previously associated with vegetative development, such as leaf development, had similar functions later elucidated in carpel development. For instance, *Arabidopsis* miR164-targeted *CUP-SHAPED COTYLEDON1* and *2* (*CUC1* and *CUC2*) - which belong to the NAC transcription factor family - regulate organ boundary during the separation between organ primordia and meristem, and control leaf margin serration (Laufs et al., 2004; Nikovics et al., 2006; Peaucelle et al., 2007; Hasson et al., 2011; Vialette-Guiraud et al., 2016). Earlier studies showed that *CUC1* and *CUC2* operate during the initial phase of organ initiation inhibiting cell growth in meristem-organ and organ-organ boundaries, facilitating the separation between adjacent vegetative and reproductive organs (Laufs et al., 2004; Mallory et al., 2004). In this process, miR164 defines boundary domains by restricting the expression of *CUC1* and *CUC2* (the miR164 module), and proper miR164 dosage and/or expression localization is required for organ separation. The miR164 module also operates further in organ development, when organ shape is being determined (Nikovics et al., 2006). In the margins of leaf primordia, *CUC2* and *MIR164A* are spatially and temporally co-expressed, and the balance between their expression controls the degree of *Arabidopsis* leaf margin serration (Nikovics et al., 2006). This module operates similarly in the regulation of leaf complexity in tomato, in which the *CUC2* ortholog miR164-targeted *GOBLET* (*GOB*) plays similar roles during boundary establishment leading to leaflet separation. Interestingly, the regulation of compound leaf development by the miR164 module is conserved in *Aquilegia caerulea*, *Solanum tuberosum*, *Cardamine hirsuta*, and *Pisum sativum* (Blein et al., 2008).

Like its function in leaf development, the miR164 module is also expressed in the margins of carpel primordium during *Arabidopsis* gynoecium development, and it determines important morphological characteristics of the mature fruit (Ishida et al., 2000; Sieber et al., 2007; Nahar et al., 2012; Kamiuchi et al., 2014; Vialette-Guiraud et al., 2016). *Arabidopsis* gynoecium is formed by two carpels that are already initiated as two fused structures, except by the apical margins, which are fused later to form style and stigma (Sessions and Zambryski, 1995; Nahar et al., 2012). During early gynoecia development, the meristematic tissue called Carpel Margin

Meristem (CMM) is originated in the margins of each carpel primordia and is responsible for producing the ovules, the ovary septum, the transmitting tract, and promoting fusion between the apical carpel margins (Alvarez and Smyth, 1999; Nahar et al., 2012; Vialette-Guiraud et al., 2016). Earlier studies showed that *CUC1* and *CUC2* expression is required for the activation of the *KNOX type-I* gene *SHOOT MERISTEMLESS* (*STM*) in different developmental contexts, such as the formation of shoot apical meristem during embryo development and leaf serration in *Arabidopsis* (Takada et al., 2001). In such processes, *STM* expression is required to establish and maintain meristematic tissues. The same mechanism seems to operate in the establishment and maintenance of CMMs during carpel development in *Arabidopsis* (Kamiuchi et al., 2014). Most *cuc1cuc2* double mutants failed to form CMM, producing mature gynoecia with drastically reduced or complete loss of ovules and septum. *Arabidopsis* plants expressing miR164-resistant versions of *CUC1* and *CUC2* showed expanded domain of *STM* expression, resulting in carpel primordia with altered size and number of CMM, of which most initiated in altered positions. These plants produce mature fruits with internal filamentous structures (Kamiuchi et al., 2014). When not regulated by miR164, *CUC1/2* expression is less precise and can expand out of the boundary strips, resulting in incorrect CMM positioning, which leads to carpel and fruit developmental aberrations.

SPATULA (*SPT*) encodes a basic helix-loop-helix (bHLH) transcription factor, and *Arabidopsis* loss-of-function *spt* mutants produce ovaries with split or incomplete fused carpels and defective CMM-derived tissues (Heisler et al., 2001; Nahar et al., 2012). *cuc1;cuc2* mutations partially suppress the split carpel phenotype of *spt* mutant, indicating that congenital carpel fusion depends on *SPT*-based down-regulation of *CUC1* and *CUC2*. Thus, the coordinated interaction among *SPT*, *CUC1*, and *CUC2* regulates *Arabidopsis* ovule and septum development during the progression of fruit growth (Nahar et al., 2012). It was recently shown that SPT enables cytokinin signalling, which provides meristematic properties to CMM. *SPT* seems to play a role in the interaction between auxin and cytokinin pathways, as *SPT* induces *ARABIDOPSIS RESPONSE REGULATOR 1* (*ARR1*) directly. *SPT* and *ARR1* induce the expression of the auxin transporter *PIN-FORMED 3* (*PIN3*) and the auxin biosynthesis gene *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1,* Reyes-Olalde et al., 2017).

The role of the tomato *CUC2* homolog *GOB* was studied in detail during leaf development and complexity, although little is known about the function of *GOB* in reproductive development. Loss-of-function *GOB* mutant (*gob-3*) produces fruits with fewer locules, whereas gain-of-function *GOB* mutant (which contains a miR164-resistant version of *GOB,* the *Gob-4d*) displays fruits with extra carpels and increased number of locules (Berger et al., 2009). Since leaf

complexity was the main objective of this work, no mechanism was proposed of how the miR164 node (miR164-targeted *GOB*) controls locule number in tomato fruits. On the other hand, tomato miR164-targeted *NO APICAL MERISTEM 2* (*SlNAM2*), another member of the NAC transcription factor family, was shown to have an important role in organ boundary maintenance during floral development (Hendelman et al., 2013). Unlike *GOB*, *SlNAM2* is not expressed in boundaries between floral meristem and organ primordia, as *SlNAM2* expression was not detected before carpel fusion in flower buds. Data thus far suggest that *GOB* functions during the formation of the boundaries, being expressed at earlier stages of organ primordia development, whereas *SlNAM2* is expressed at later stages of floral whorl development, being responsible for the maintenance of the boundaries established by *GOB* (Hendelman et al., 2013). Plants overexpressing *mSlNAM2* (a miR164-resistant version of *SlNAM2*) produced gynoecia with shorter stamen and styles and wide pistil, the latter likely due to the extra carpel formation. Although weaker, *mSlNAM2* phenotypes were similar to *Gob-4d* phenotypes, which is consistent with the proposed *SlNAM2* role in boundary maintenance, but not boundary formation (Berger et al., 2009; Hendelman et al., 2013). In summary, the functions of the miR164 module in *Arabidopsis* and tomato gynoecium patterning illustrates the crucial importance of boundary formation and maintenance during fruit development. Proper function of the miR164 module is essential for the establishment and maintenance of gynoecium development, not only in syncarpous species such as *Arabidopsis* and tomato, but also in monocarpous species like *Medicago trunculata* (Berger et al., 2009; Vialette-Guiraud et al., 2016).

1.2.2. The role of miR156/miR157 in carpel and fruit development

MiR156 targets members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SBP/SPL*) transcription factor family. In *Arabidopsis* and tomato, 11 out of 17 *SBP/SPL*s harbor the miR156 recognition site (Salinas et al., 2012; Preston and Hileman, 2013). The miR156 module (miR156-targeted *SBP/SPLs*) defines the evolutionary conserved age-dependent floral pathway in several plants, including tomato (Silva et al., 2018). Interestingly, the miR156 module has been proposed as a main target for crop improvement, aiming to enhance agronomic traits such as the timing of vegetative and reproductive phase change, leaf development, tillering/branching, panicle/tassel architecture, fruit development and fertility (Wang et al., 2015).

In terms of gynoecium and fruit development, it was demonstrated that *Arabidopsis SPL8* (which is not targeted by miR156) acts redundantly with miR156-targeted *SPLs* in the control of carpel development (Xing et al., 2013). Transgenic plants overexpressing miR156

(*p35S::MIR156b*) produce flowers with reduced ovary size but unaffected structure, while ovaries of *spl8-1* mutant show a slight reduction in size and resembles wild-type (WT). Conversely, the double mutant *p35S::MIR156b spl8-1* show extremely modified gynoecia. The gynoecium shape of *p35S::MIR156b spl8-1* is completely altered, displaying an enlarged upper region and a narrower basal region, abnormal septum development, and absence of transmitting tissue to support pollen tube growth into the ovary (Xing et al., 2013). Considering that *SPL8* and the miR156-targeted *SPLs 2, 6, 10, 11 and 13* are expressed in overlapping domains during gynoecium development, this data supports the idea that they have partly redundant roles in the patterning of the gynoecium and fruit development. Furthermore, seed production decreased about 60% in *p35S::MIR156b* plants in comparison with WT and *spl8-1* (which show unaltered seed production), whereas *p35S::MIR156b spl8-1* produces approximately 96% less seeds than WT (Xing et al., 2013). Together, these data indicate that the function of at least one of these *SPLs* is crucial for proper gynoecia development. Another study showed that *Arabidopsis squint* (*sqn*) mutants contain loss-of-function alleles for *cyclophylin40* (*CyP40*), which increases the activity of miR156 by promoting *AGO1* activity. *sqn* plants showed elevated expression of miR156-targeted *SPL*s and produce siliques with increased carpel number (Smith et al., 2009).

Interestingly, the miR156 module may function by different mechanisms or have different roles in dry fruit and fleshy fruit-bearing species. As mentioned above, ovaries of *Arabidopsis p35S:MIR156b* plants do not present extra carpels or undetermined growth (Xing et al., 2013). On the other hand, the overexpression of miR156 (*p35S:MIR156b*) in tomato plants led to the production of extremely modified ovaries formed by multiple fused extra carpels and undifferentiated tissue inside the post-anthesis ovaries (Silva et al., 2014). After fruit set, the undifferentiated tissue inside the ovaries of *p35S:MIR156b* plants continues to grow, forming fruit-like structures growing from the stylar end of the fruits. Furthermore, mature fruits show increased number of locules due to the presence of extra carpels in the ovary (**Figure 1**; Silva et al., 2014). Floral identity genes like *FUL1/TDR4*, *FALSIFLORA* (*FA*, *Arabidopsis LEAFY* ortholog; Lozano et al., 2009) and *MACROCALLYX* (*MC*, *Arabidopsis APETALA1* ortholog; Lozano et al., 2009) were strongly down-regulated in tomato *p35S:MIR156b* ovaries (Silva et al., 2014). *Arabidopsis FUL*, *AP1* and *LFY* are direct targets of SPL3 (Yamaguchi et al., 2009), although it is still unknown whether their tomato orthologs are direct targets of SlSBP3. Interestingly, the *CUC2* and *STM* orthologs *GOB* and *TKN2,* respectively, are up-regulated in tomato *p35S:MIR156b* ovaries. MiR164-targeted *GOB* and *TKN2* are associated with leaf complexity in tomato but both can also regulate the number of locules per fruit (Parnis et al., 1997; Berger et al., 2009). This finding indicates a link between miR156 and miR164 modules and

suggests that tomato miR156 module controls boundary formation and establishment as well as locule number through *GOB* and perhaps other NAC domain-containing genes (such as *SlNAM2*). As expected, tomato plants overexpressing miR164 lead to *GOB* down-regulation and the production of fruits with normal shape but reduced locule number (Silva et al., 2014).

Most plant genomes also contain miR157, a miR156 closely related miRNA which differs from miR156 by three nucleotides (Reinhart et al., 2002). MiR157 overexpression in *Arabidopsis* generates plants phenotypically similar to miR156 overexpressors, but miR157 specific functions are still unknown (He et al., 2018). MiR157 seems to be more abundant but less effective on *SBP/SPL* repression, perhaps because it is less efficiently loaded onto *AGO1* (He et al., 2018). Transgenic cotton plants overexpressing miR157 produced smaller gynoecium, with less ovules per ovary and decreased seed production in comparison with WT (Liu et al., 2017). These plants showed reduced expression of two MADS-box transcription factors (orthologs of *AtAGL6* and *SlTDR8*). In addition, auxin response was attenuated in ovaries of miR157-overexpressing cotton plants. It is possible that miR156 and miR157 modules regulate gynoecium development by overlapping but also specific mechanisms, although additional studies are needed to unravel miR157 specific functions in reproductive development.

1.2.3. miR396 module regulate CMM meristematic competence and pluripotency during gynoecium development

The *GROWTH-REGULATING FACTORs* (*GRFs*) belong to a plant-specific transcription factor family that has nine members in *Arabidopsis*, seven of which (*GRF1*, *GRF2*, *GRF3*, *GRF4*, *GRF7*, *GRF8*, and *GRF9*) are targeted by miR396, representing the miR396 module (Lee et al., 2014; Liang et al., 2014; Lee et al., 2017). MiR396 module regulates several developmental processes, such as leaf development, floral development, and root cell reprograming during nematode infection (Lee et al., 2009; Hewezi et al., 2012).

Arabidopsis plants overexpressing miR396 (*p35S:MIR396a*) show gynoecium developmental defects such as gynoecia formed by only one carpel and siliques (dry fruits) producing a reduced number of seeds (Liang et al., 2014; Lee et al., 2017). *Arabidopsis* GRFs interact physically in the nucleus with the transcriptional co-activators GRF-INTERACTING FACTOR1, 2 and 3 (GIF1, GIF2 and GIF3) (Liang et al., 2014). Because GIF-GRF complexes are crucial for meristematic competency and pluripotency of CMM cells (Lee et al., 2017), high miR396 levels may lead to low *GRF*s available to form these heterodimers, hence CMM loses its meristematic competence and pluripotency over time (Liang et al., 2014; Lee et al., 2017). Single

GRF loss-of-function mutants produce WT-like siliques, whereas g*if1* single mutant produces normal pistil and siliques but with reduced size. Siliques of the double transgenics *p35S:MIR396a ;p35S:mGRF7* and *p35S:MIR396a ;p35S:mGRF9* (both expressing miR396-resistant versions of *GRF7* and *9* transcripts, respectively) can recover WT silique phenotypes, indicating that at least miR396-targeted *GRF7* and *9* have roles in fruit development (Liang et al., 2014). The phenotypes of gynoecium and siliques of the triple mutant *gif1 gif2 gif3* phenocopy those of the double mutant *p35S:MIR396a grf5* (*GRF5* is not targeted by miR396), producing extremely short and almost sterile siliques, generally lacking valves, whereas some *GRF* triple mutants (*e.g. grf1/grf2/grf3* and *grf7/grf8/grf9*) present WT-like siliques (Liang et al., 2014). The triple mutant *grf1 grf3 grf*5 show single-valve gynoecia and slight defects on floral organ separation and number, but these defects were strongly enhanced by the addition of *grf2* mutation to this background (generating the quadruple mutant *grf1grf2 grf3 grf5*). Together, these findings indicate that *GRFs* act redundantly to modulate *Arabidopsis* gynoecium patterning and fruit development.

The mechanisms by which GRF-GIF dimers promote CMM meristematic capacity in *Arabidopsis* gynoecium were not well elucidated, but available data suggest that they may be associated with polar auxin transport (PAT) (Lee et al., 2017). *Arabidopsis* PAT mutants (*pin-formed1* and *pid*) and some auxin biosynthesis mutants (*yuc1*, *yuc4* and *wei8 tar2*) produce gynoecia phenotypes identical to *gif p35S:MIR396a* plants and *grf* multiple mutants. The addition of *gif* mutations to a *pid-3* mutant (a *PINOID* mutant with weak developmental defects) or treatment of *gif* mutants with N-1-Naphthylphthalamic Acid (NPA, an auxin polar transport inhibitor) synergistically enhance gynoecium developmental defects of *pid-3* or NPA-treated WT plants (Lee et al., 2017). These findings indicate an interplay between miR396, *GRF-INTERACTING FACTORs* and auxin during gynoecium patterning.

Unlike *Arabidopsis*, the possible role of the miR396 module in tomato fruit development has not been described in detail. The only study in tomato thus far showed that miR396 down-regulation (or *GRF* de-regulation) seems not to affect CMM formation but rather it leads to a significant increase in fruit size (Cao et al., 2016). This is consistent with the main role of GRFs in modulating cell proliferation and cell expansion in several developmental contexts (Lee et al., 2009). Since neither fruit shape nor ripening was altered in the transgenic tomato plants down-regulating miR396 (Cao et al., 2016), the authors proposed that these plants might provide a new way to enhance tomato fruit yield.

1.2.4. microRNA160 module controls carpel development by modulating auxin responses

Some microRNAs, such as miR160, are crucial for auxin signaling during several developmental processes. MiR160, which targets the AUXIN RESPONSE FACTORS *ARF10*, *16* and *17* (Hendelman et al., 2012; Damodharan et al., 2016; Li et al, 2016), is another example of a miRNA module that apparently has different roles in the regulation of dry and fleshy fruit development.

The *Arabidopsis floral organs in carpels* (*foc*) mutant contains a *Ds* transposon insertion in the 3´regulatory region of the *MIR160a* gene, which disrupts its native expression pattern, leading to the accumulation of *ARF10*, *16* and *17* and low auxin responses in various organs (Liu et al., 2010). These regulatory disruptions lead to abnormal embryo, seed, and flower development. *foc* plants show some degree of indeterminacy during gynoecium patterning, which is observed by the production of floral organs inside the siliques and sometimes whole inflorescences emerging from siliques. Furthermore, *foc* mutant produces abnormal seeds and viviparous seedlings. It was also shown that 3´regulatory region bears three putative auxin-responsive elements (AuxRE) and *MIR160a* expression is positively regulated by auxin. Thus, the disruption of this regulatory region impairs the induction of *MIR160a* expression by auxin, impacting fruit development (Liu et al., 2010).

The miR160 module (miR160 and their targets) seems also to have an important, but different, role in tomato fruit development. Transgenic tomato plants (*STTM160*-expressing plants) with knocked-down miR160 expression generated by the Short Tandem Target Mimic (STTM) approach (Teotia and Tang, 2017) produce ovaries with elongated morphology and thinning of the placenta, which developed into fruits with abnormal pear-shaped fruit morphology. These changes were associated with miR160 depletion and concomitant de-regulation of *SlARF10B* and *SlARF17*, and mostly *SlARF10A* in *STTM160*-expressing plants (Damodharan et al., 2016). Nevertheless, unlike *Arabidopsis foc* mutant, no indeterminacy was observed in gynoecia of *STTM160*-expressing tomato plants. Such discrepancy between phenotypes of tomato and *Arabidopsis* miR160 loss-of-function plants may be due to the fact that *SlARF16* is not de-regulated in *STTM160*-expressing tomato plants, despite the miR160 legitimate site observed in *SlARF16* (Damodharan et al., 2016).

MiR160-guided cleavage of some ARFs is also needed for proper leaf development in tomato and Arabidopsis. Interestingly, *STTM160* tomato plants and *5mARF17* (plants expressing a miR160-resistant version of *ARF17*) Arabidopsis plants showed similar leaf

phenotype, which is reduced leaf blade and strongly lobbed leaflet/leaf margins (Mallory et al., 2005; Damodharan et al., 2016).

1.2.5. miR172 limits the growth-repressing activity of *APETALA2*-like genes during fruit expansion

All microRNA modules discussed so far are mostly associated with very early stages of carpel development, such as patterning and differentiation, and the proper control of these stages have great impact on mature fruit morphology and fertility. On the other hand, the miR172 module seems to control not only fruit patterning, but also fruit growth, which comprises a developmental stage after pollination, when the ovary is fully developed. In *Arabidopsis*, the miR172 module comprises the microRNA172 and its targets (*APETALA2-LIKE* (*AP2-like*) transcription factors): *APETALA2* (*AP2*), *TARGET OF EAT1, 2* and *3* (*TOE1*, *TOE2*, and *TOE3*), *SCHLAFMUTZE* (SMZ), and *SCHNARCHZAPFEN* (*SNZ*) (Wu et al., 2010). Interestingly, pioneer studies showed that miR172 can guide not only *AP2-like* transcript degradation but also its translational repression (Chen, 2004).

Arabidopsis fruit undergoes dramatic increase in fruit size after fertilization, and different tissues grow at different rates (for review please see Ferrándiz et al., 2010). MiR172 module seems to be crucial to specify which regions of the carpel will go through dramatic expansion and which region will arrest fruit growth. *AP2* encodes an AP2/EREBP transcriptional repressor, which was shown to repress valve margin and replum growth post-fertilization by repressing the expression of genes that confer identity to valve margin (*INDHEISCENT* and *SHATTERPROOF*) and replum (*BREVIPEDICELLUS* and *REPLUMLESS*) (Ripoll et al., 2011). In this context, *AP2* prevents replum overgrowth and overproliferation of the layer of lignified cells (LL) (which are associated with fruit dehiscence; Rajani and Sundaresan et al., 2001; Liljegren et al., 2004) in the valve margin. Consistent with this, *ap2* mutants produce siliques with oversized replum and slightly delayed dehiscence due to increased number and size of LL (Ripoll et al., 2011). Nevertheless, after pollination the valves undergo a conspicuous cell expansion stage, increasing dramatically fruit size. This pollination-dependent valve growth was shown to be blocked in plants with decreased miR172 activity - via target mimicry (*MIM172*) approach (Franco-Zorrilla et al., 2007) - and in plants expressing a miR172-resistant *AP2* version, resulting in smaller fruits (Ripoll et al., 2015). For proper valve expansion, *AP2* and *TOE3* activities must be inhibited by miR172 only in the valves. The MAD-box transcription factor *FRUITFULL* (*FUL*) displays similar expression pattern as miR172, being expressed in the valves, and *ful*

mutants resemble *MIM172* plants, presenting arrested growth phenotype in the valves. Furthermore, analysis of different degrees of homo and heterozygosity of *ARF6* and *ARF8* mutant alleles *arf6* and *arf8* in double mutants show that fruit valve expansion decreases with the increasing *ARF* mutant allelic dosage. Valve growth is even more limited when *arf6/8* are introduced in *ful*, and *arf6 arf8 ful* triple mutants produce siliques with extremely impaired growth. *FUL*, *ARF6*, and *ARF8* are expressed only in the valves (except valve margins), where they form protein complexes that bind to the *MIR172C* promoter and activate its expression. *AP2* and *TOE3* are expressed in the whole carpel, but miR172 induction in the valves restricts *AP2* activity to the valve margins and replum, allowing it to repress cell elongation in these locations but not in the valves. Through this mechanism, miR172 fine-tunes fruit patterning and growth by restricting the activity of *AP2-like* genes to certain locations within the fruit (Ripoll et al., 2015). Considering that miR167 negatively regulates *ARF6* and *ARF8* (Wu et al., 2006), it will be interesting to determine whether this miRNA participates in this mechanism by specifying *ARF6/8* expression pattern.

Although high levels of miR172 have a positive effect on *Arabidopsis* fruit growth (Ripoll et al., 2015), this is not always the case for other species. For instance, over-expression of a *MIR172* gene has a negative influence on fruit growth in apple (*Malus domestica*), resulting in a dramatic reduction in fruit size (Yao et al., 2016). Unlike *Arabidopsis* and tomato fruits, which are both derived from ovaries, apple fruits are mostly derived from the hypanthium that is hypothesized to consist of the fused bases of the sepals, petals and stamens (Pratt, 1988). Interestingly, over-expression of the same *MIR172* gene in tomato results in carpel-only flowers which developed into parthenocarpic fruits (Yao et al., 2016). These examples nicely illustrate that the influence of a particular miRNA module on fruit growth depends on the fruit type and plant species.

1.3. MicroRNA-controlled pathways modulating ovule and seed development during fruit growth

The ovule is the female sexual organ in higher plants and a strict control of ovule development is crucial for plant reproductive success. Ovule is required to enclose the female gametophytes and, more importantly, it is from the fertilized ovules that seeds arise. Ovule structures are conserved in most plants, and comprise the embryo sac, the nucellus, the integument (which originates the seed coat) and the funiculus, which makes the connection between the ovule and placenta. Ovule and seed development are under control of genetic (e.g., transcription factors, non-coding RNAs), physiological (hormones) and epigenetic factors (i.e.

chromatin remodelling and DNA methylation) (Skinner et al., 2004; Kelley and Gasser, 2009; Yamaguchi et al., 2013; Cucinotta et al., 2014). In this part of the review, we will discuss the findings of how some small RNAs modules act to modulate ovule and seed development, which are crucial developmental processes that take place during fruit development and ripening.

It was recently shown by our research group that the miR159 module is crucial for ovule and seed development in tomato (Silva et al., 2017). The miR159 module comprises the microRNA159 and its targets, *SlGAMYB1* and *SlGAMYB2*, which belong to the R2R3 MYB domain transcription factor family. *GAMYB-like* genes are regulated by gibberellin and by the microRNA159 family in different tissues and developmental contexts (Gubler et al., 1995; Tsuji et al., 2006; Alonso-Peral et al., 2010). MiR159 and its targets are expressed early during tomato placenta and ovule development, which suggest that the miR159 module may be involved in the initial steps of ovule development. Likewise, the overexpression of *SlMIR159* (*p35S::SlMIR159*) disrupts ovule development and induces obligatory parthenocarpy. Such phenotype is more severe than what is shown in *AtMIR159a*-overexpressing Arabidopsis plants, which generates fertile siliques when pollinated with WT pollen (Achard et al., 2004). Tomato, transgenic plants harboring the *p35S::SlMIR159* construct displays defects in the establishment of the embryo sac, which may be due to the observed lower expression of *AINTEGUMENTA*-like genes (Silva et al., 2017). *AINTEGUMENTA* (*ANT*) gene is an *APETALA2-like* transcription factor required for ovule and integument initiation (Elliott et al., 1996). Although tomato lacks known *ANT* mutants, it was shown in rice that *ANT* was also strongly repressed in *gamyb* mutants displaying ovule developmental defects (Tsuji et al., 2006). MiR159 module interacts with tomato *AINTEGUMENTA-like* genes to drive developmental progression of ovules and, thus, modulates tomato fruit set. Moreover, our work showed that miR159 module interacts with the miR167 module. Down-regulation of miR167 and concomitant *SlARF8* de-regulation in *p35S::MIR159* plants may be also responsible for the arrested ovule development (Silva et al., 2017), illustrating the link between the miR159 module and auxin during fruit set.

Parthenocarpy, the developmental process in which fruits develop in the absence of fertilization (Varoquaux et al., 2000), can be easily induced in grapevine (*Vitis vinifera*) by exogenous gibberellin (GA) application (Wang et al., 2018). These authors show that *Vvmi*R*159c* and its target *VvGAMYB* are dynamically and opposing expressed during flowering and fruit set. GA treatment is capable of inducing *VvmiR159c* and, consequently, down-regulating *VvGAMYB* in reproductive organs. These observations led the authors to suggest that the miR159 module is associated with GA-induced parthenocarpy in grapevine (Wang et al., 2018), similarly to what we have discovered in tomato (Silva et al., 2017).

The use of high-throughput sequencing approaches also provided evidences of the activity of miRNA modules during ovule development. In cotton (*Gossypium hirsutum*), small RNAs profiles of developing ovaries showed distribution of several small RNA signatures, including microRNAs (Abdurakhmonov et al., 2008). Several conserved microRNA families were identified in cotton ovules, including miR156/157, miR159, miR164, miR168, and miR395. These results are important to provide initial information for future functional experiments. In addition, several predicted miRNA targets were validated via degradome sequencing (a modified version of 5'-Rapid Amplification of cDNA Ends that is combined with high-throughput, deep sequencing to detect transcript ends; Ma et al., 2015), reinforcing the idea that conserved miRNA modules may be important in ovule development of cotton (Xie et al., 2015).

MicroRNAs are also required for embryogenesis, which is a key developmental step for plants to establish the seed set. To complete its development, the embryo undergoes specific stages, which in *Arabidopsis* are defined by its morphology as globular, heart, torpedo, and walking stick stages (Jürgens, 2001). Such developmental stages are known to be regulated by transcription factors, small regulatory RNAs, signal transduction orchestrated by kinases, auxin gradients, and epigenetic mechanisms (*i.e.* DNA methylation, histone acetylation, among others). Thus, these regulatory pathways are key determinants of the fate of primordia cell lineages, and also drive inheritance that is programmed via mitosis at early stages of the embryo development (Willemsen and Scheres, 2004).

DICER-LIKE1 (DCL1) is a key enzyme for the pri-/pre-miRNA processing (Park et al. 2002; Reinhart et al. 2002; Kurihara and Watanabe 2004). Genome-wide transcriptional profiling of the *Arabidopsis* mutant *dicer1 (dcl1)* shed some light regarding the importance of microRNA modules during early embryo development. At the early globular stage, *dcl1* embryo display about 50 miRNA targets de-repressed due to the lack of miRNA regulation. Some of these targets (usually transcription factors) are required for differentiation at later stages of embryogenesis (Nodine and Bartel 2010). In addition, in *dcl1* embryos, miR156-targeted *SPL10* and *SPL11* are highly up-regulated, which suggest that the de-regulation of these transcription factors is at least in part responsible for the *dcl1* embryo abnormalities (morphological defects and arresting growth at the globular stage). Thus, one of the first roles of plant microRNAs is to repress its targets at early developmental stages to prevent precocious differentiation during embryogenesis (Nodine and Bartel 2010). This idea is further supported by the finding that *Arabidopsis* double mutant *ago1/ago10* displays embryo lethality, probably due the highly activity of small RNAs targets (Lynn et al. 1999; Mallory et al., 2009). Argonaute (AGO) proteins are part of the RNA-induced

silencing complex (RISC), and are required for the repression of microRNA targets (Rhoades et al. 2002; Zilberman et al., 2003).

MiRNA module may also affect seed development. MiR397 negatively regulates members of the Laccase family. MiR397-targeted *Laccase4* is a member of the blue copper oxidase/ p-diphenol:dioxygen oxidoreductase family and participates in lignin biosynthesis. (Gavnholt and Larsen, 2002; Mayer and Staples, 2002). The miR397/*Laccase4* module has been implicated in the control of the number of seeds and seed size. Overexpression of *MIR397b* in *Arabidopsis* leads to reduce lignin deposition. Interestingly, in terms of fruit development, transgenic plants with less lignin produce bigger siliques with more and enlarged seeds. Similar results are observed in transgenic rice plants overexpressing *MIR397a* and *MIR397b*, which are able to produce enlarged grains (Wang et al., 2014; Zhang et al., 2013). Such studies highlight that miR397-mediated development via regulating *laccase* genes might be a potential tool not only for engineering plant biomass production with less lignin, but also for manipulating plant seed yield.

1.4. Noncoding RNAs in the regulation of fruit ripening

In the first section of this review, we discussed the main microRNA modules involved in diverse aspects of early fruit development, which is summarized in **Figure 2A**. In this last section, we will discuss a few examples available in the literature that reinforce the fundamental roles of non-coding RNA-mediated regulation also in fruit ripening.

1.4.1. Conserved and Solanaceae-specific miRNA modules control tomato fruit ripening

Tomato plants bearing the dominant mutation *Cnr* (*COLORLESS NONRIPENING*) produce fruits with characteristics associated with impaired ripening, such as inhibited softening, yellow skin, and pericarp lacking pigments because of the arrested biosynthesis of ripening-related pigments (Thompson et al., 1999). Furthermore, mutant plants produce lower amounts of ethylene and exogenous ethylene application does not recover this phenotype. Positional cloning showed later that a *SPL/SBP* gene (called *SlSBP3/CNR*) containing a potential miR156/157 binding site resides in the *Cnr* locus. *Cnr* is an epimutation caused by spontaneous heritable hypermethylation of cytosine residues of the *SlSBP3/CNR* promoter, leading to *SlSBP3/CNR* repression (Manning et al., 2006). Although the mechanism by which *SlSBP3/CNR* controls fruit ripening remains unclear, recent data suggest that the MADS-box transcription factor

RIPENING INHIBITOR (*RIN*) and *CNR* may be part of the same protein complex that induces the expression of ripening-related genes (Martel et al., 2011). *RIN* controls both ethylene-dependent and independent ripening regulatory pathways, interacting directly with the promoter of many known genes associated with key ripening processes, such as ethylene biosynthesis, perception and signal transduction, cell wall metabolism, and carotenoid biosynthesis. Nevertheless, *CNR* is required for RIN promoter binding activity, as RIN does not interact with the promoters of ripening-related genes in the *Cnr* mutant (Fujisawa et al., 2013; Martel et al., 2011; Qin et al., 2012). Although CNR and RIN proteins do not interact, it is possible that these transcription factors are part of the same protein complex that modulates the expression of key ripening genes. Substantiating this hypothesis, *rin* and *Cnr* mutants have similar fruit phenotypes such as blocked ripening and impaired response to exogenous ethylene (Vrebalov et al., 2002; Martel et al., 2011).

Virus-induced gene silencing (VIGS)-based delivery of mature miR157 in tomato fruits reduced *CNR* transcript accumulation and delayed ripening in the injected fruit areas (Chen et al., 2015). Degradome analyses indicate that miR156 cleaves *CNR* in different stages of fruit ripening (Karlova et al., 2013). Surprisingly, VIGS-based delivery of miR156 does not produce any alteration in fruit ripening until the breaker stage, and these fruits show early softening (Chen et al., 2015). These observations suggest that the miR156/miR157 module may be necessary for proper control of fruit ripening and that the closely-related miR156 and miR157 play different roles in the temporal control of the ripening-associated processes.

Tomato miR172-targeted *AP2a* appears to have complex functions in the control of diverse ripening-related processes, regulating mostly genes associated with ethylene biosynthesis and signaling (Karlova et al., 2011). *AP2a* silencing through RNAi leads to the production of fruits that ripe, but never turn from orange to red, showing altered levels of various carotenoids and increased chlorophyll levels, although they produce high levels of ethylene. *AP2a* seems to act downstream to *RIN* and *CNR*, as its expression is negatively regulated in *rin* and *Cnr* mutants and CNR binds to *AP2a* promoter. Thus, *CNR* induces *AP2a* expression directly, although *AP2a* represses *CNR* expression in a negative feedback loop (Karlova et al., 2011). Taken together, the evidences in tomato thus far indicate that both miR156/miR157 and miR172 modules and the interaction between their targets (*CNR* and *AP2a*) are important to proper fruit ripening. In fact, degradome analysis showed that levels of the *CNR* and *AP2a* miRNA-guided cleavage products vary among different ripening stages, showing peak accumulation of cleavage transcripts during breaker stage, which is also the peak of ethylene production (Karlova et al., 2013). It will be

interesting to determine whether these miRNAs have specific roles in fine-tuning spatially and/or temporally the expression of their targets during fruit ripening.

Recently, a novel miRNA identified as Solanaceae-specific was implicated in regulating ethylene signaling and hence fruit ripening in tomato (Wang et al., 2018). The microRNA miR1917 targets three splicing variants of the *CONSTITUTIVE TRIPLE RESPONSE 4* (*SlCTR4*, homolog of *Arabidopsis CTR1*), an ethylene signaling repressor that interacts with ethylene receptors (Wang et al., 2018). Tomato plants overexpressing the miR1917 (*p35S::MIR1917*) display higher levels of ethylene signaling, leading to enhanced ethylene production. These plants also have increased ethylene responses in the absence of ethylene, including accelerated pedicel abscission and fruit ripening (Wang et al., 2018). The complementary expression pattern of miR1917 and the splicing variants *SlCTR4*sv3 observed in the pedicel abscission zone by *in situ* hybridization suggests that miR1917 restricts the expression of its targets to the vascular bundle and surrounding cells during pedicel abscission. Thus, miR1917 and its targets represent a novel miRNA module belonging to the intricate ethylene-associated signaling network.

1.4.2. New evidences of the role of long non-coding RNAs (lncRNAs) in fruit ripening

Long non-coding RNAs are broadly present in plant, animal and fungi transcriptomes and emerging evidences show that they play key roles in diverse developmental processes. They are RNAs longer than 200 nt originated from transcription of intergenic regions, introns or antisense coding sequences and do not have any detecting coding potential (Chekanova et al., 2007; Chekanova, 2015; Fatica and Bozzoni, 2014; Kapranov et al., 2007). LncRNAs may modulate gene expression by multiple mechanisms that were extensively reviewed in Chekanova (2015). Although the knowledge of the regulatory roles of lncRNAs in plants is still limited, lncRNAs have been associated with the control of flowering time, male sterility, seedling morphogenesis and, more recently, fruit ripening (Berry and Dean, 2015; Ding et al., 2012; Li et al., 2018; Wang et al., 2014).

RNA-seq analyses comparing transcriptomes of tomato cv Ailsa Craig and *rin* fruits identified over 3000 tomato lncRNAs, several of which were differentially expressed in *rin* (Zhu et al., 2015). In the same study, two lncRNAs (lncRNA1459 and lncRNA1840) strongly down-regulated in *rin* were chosen for VIGS-based silencing assays in fruits. Silencing of both lncRNAs produced non-ripening sections in the injected areas of the fruit, similarly to the effect observed

in VIGS-based silencing of *RIN*. To better understand the functional role of lncRNA1459, which is a sense intergenic lncRNA, Li et al. (2018) generated loss-of-function mutants for lncRNA1459 using clustered regularly interspaced short palindromic repeats (CRISPR)/-associated protein 9 (Cas9)-induced genome editing technology (Feng et al., 2013; Doudna and Charpentier, 2014). Mutant fruits display delayed ripening phenotype associated with repressed ethylene and carotenoid biosynthesis, as well as down-regulation of ripening-associated genes.

In addition to tomato, lncRNAs involved in fruit ripening have been identified and studied in few other species. Sea buckthorn (*Hippophae rhamnoides*) is a plant for

land reclamation, and its berry-type fruits have high nutritional value due to the significant amounts of natural anti-oxidants including ascorbic acid, tocopherols, carotenoids, and flavonoids (Zakynthinos et al., 2016). By using high throughput RNA sequencing, Zhang et al. (2018) identified over 9000 lncRNAs expressed in distinct sea buckthorn fruit developmental stages, from mature green to red-ripe. Interesting, the authors identified two lncRNAs (LNC1 and LNC2) that may function as target mimics of miR156 and miR828 during fruit ripening, therefore indirectly affecting the expression of these miRNA targets, *SPL9* and *MYB114*, respectively. By modulating *SPL9* and *MYB114* expression, LNC1 and LNC2 seem to control the biosynthesis of anthocyanin during fruit ripening (Zhang et al., 2018).

Despite the examples given above, the functions of the majority of ripening-associated lncRNAs are still unclear. More functional studies are needed to confirm the function of lncRNAs and their possible target genes. On possibility to be further explored is that lncRNAs can interact with microRNAs to modulate gene expression level (Gorospe et al., 2014), thus combining the "power" of two ncRNAs to modulate fruit ripening (**Figure 2B**).

1.5. Conclusion

During plant development, multiple microRNA modules are required to control meristem identity, leaf margin serration, polarity, complexity, root development, and flowering time. As summarized here, miRNA modules have key roles in fruit development, ranging from carpel establishment and patterning to fruit ripening. Disruption of miRNA transcription or processing frequently generate pleotropic consequences for the plant. Indeed, their activity are essential for plants to complete their life cycle, since they are active from seed to flower production. Interestingly, evolution of miRNA modules brought about adaptative advantages to plants by using similar pathways to orchestrate different developmental processes. A good example presented here is the miR164 module, which is required for proper leaf and carpel/fruit development, corroborating the hypothesis of the evolutionary origin of carpel as modified

leaves. It is interesting to consider that evolution has also hijacked similar microRNAs modules to control unrelated developmental programs such as the role of the miR156 module in flowering time and fruit development and ripening. In addition, due to their multiple roles in plant development, microRNA modules may also provide promising molecular tools to be explored in an agricultural context. Therefore, the better understanding of the mechanisms that control miRNA and target expression and their spatiotemporal regulatory roles could be an outstanding step towards the application of microRNA-targeted regulation of important fruit traits, including size, shape, seed production, and ripening. For instance, the use of novel CRISPR/Cas9-based technologies (Li et al., 2018) might allow subtle changes in miRNA target gene expression which have a potential to quantitative modify fruit traits. Additionally, it would be interesting to investigate whether there are more specific microRNAs modules (e.g., Solanaceae-specific microRNA mentioned in this review) in others crops that might be associated with fruit quality traits. Although there are open questions of how microRNA modules function during fruit development, lncRNA-associated pathways are probably one of the less understood so far, involving multiple and complex origins and modes of action. As mentioned in this review, microRNAs and lncRNAs act during fruit ripening, and they can interact during this process. In addition, since they may have overlapping functions during ripening, it would be interesting to investigate whether these two classes of non-coding RNAs interact in early steps of carpel development and fruit patterning as well. The identification of additional lncRNAs and miRNAs and the understanding of how they interact with each other to control fruit development and ripening would be an important step towards the improvement of fruit production. The use of next generation sequencing technologies combined with functional genomics may help to achieve this goal.

Author contributions: EMS, JPOC, and FTSN conceived the review. EMS and JPOC wrote the review. FTSN helped writing and correcting the final version of the review.

Acknowledgements: We thank Dr. Nogueira's lab members for helpful discussions. EMS and JPOC were the recipient of a State of Sao Paulo Research Foundation (FAPESP) fellowships nos. 13/02469-6 and 16/05073-4, respectively. This work was supported by FAPESP (grant no. 15/17892-7).

Conflict of Interest: the authors declare that they have no conflict of interest.

Figures

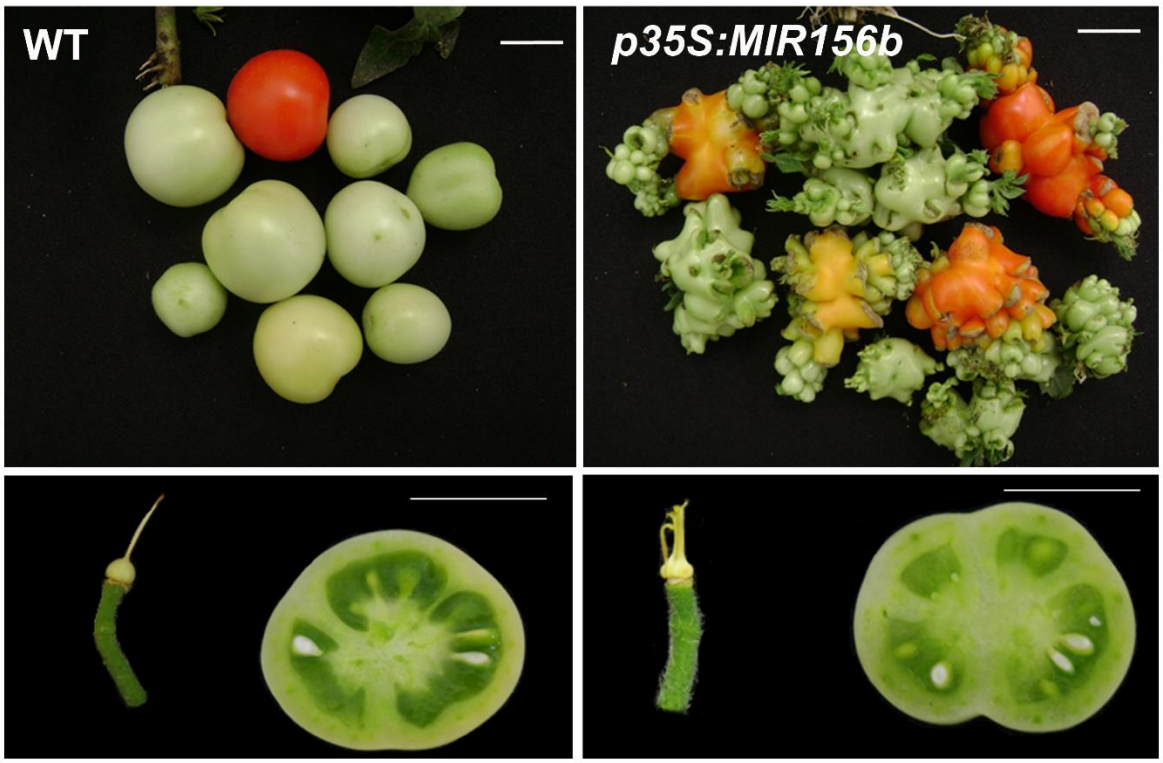

Figure 1. *MIR156* overexpression alters tomato fruit determinacy and locule number. Upper panels: wild-type (WT) fruits and undetermined *p35S::MIR156b* fruits. Low panels: three-locular WT fruits and four-locular *p35S::MIR156b* fruits. Bars: 1 cm.

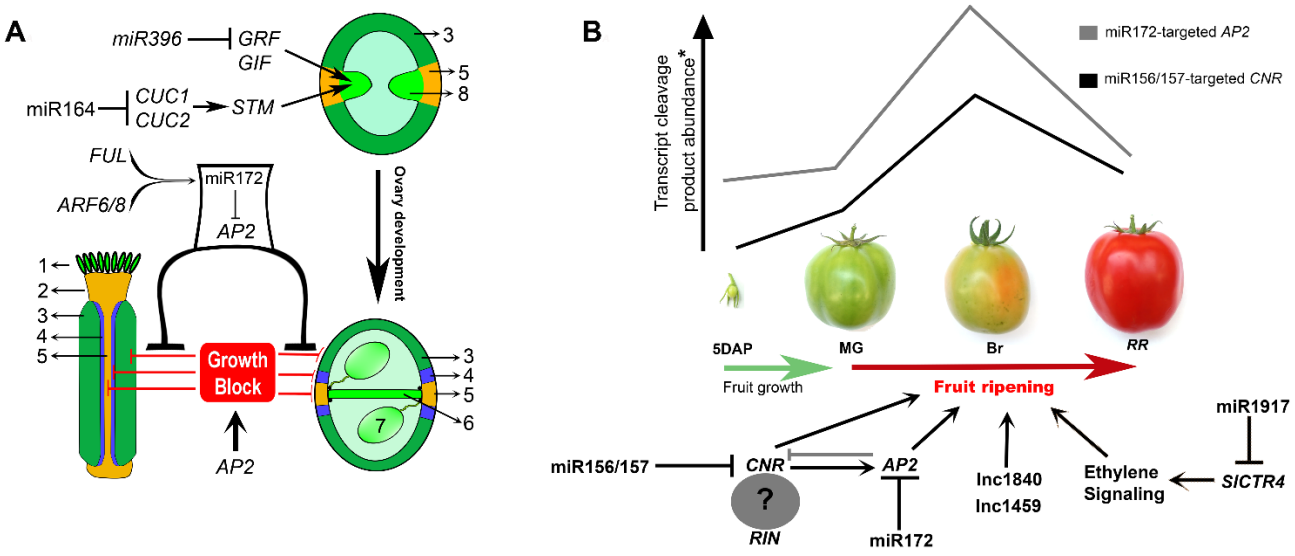

Figure 2. Non-coding RNA networks associated with carpel patterning and fruit ripening. (A) Summary of miRNA modules that control early (e.g., CMM establishment and maintenance) and late aspects of *Arabidopsis* fruit development. MiR396 and miR164 modules have important regulatory roles in CMM maintenance. *MIR172C* is induced by AUXIN RESPONSE FATORS ARF6/8 and FRUITFULL (FUL) specifically in the valves, and this specificity is necessary for proper fruit growth after pollination. MiR172-guided *APETALA2* (AP2) mRNA cleavage in the valves (but not valve margins) promotes valve growth due to the repression of *AP2* growth-blocking activity. Growth is blocked by *AP2* in valve margins and replum, where miR172 is not expressed. 1 - stigma, 2 - style, 3 - valve, 4 - Valve margin, 5 - replum, 6 - septum, 7 - ovule, 8 - carpel margin meristem (CMM). GRF, GROWTH-REGULATING FACTOR; GIF, GRF-INTERACTING FACTOR; STM, SHOOT MERISTEMLESS. (B) Graphic shows the accumulation of miRNA-cleaved transcripts of *CNR* and *AP2* through four stages of fruit development/ripening: 5 days after pollination (5 DAP), Mature green (MG), Breaker (Br) and Red ripe (RR) (adapted from Karlova et al., 2013). MRNA cleaved product accumulation occurs in the breaker stage, coinciding with an ethylene peak production. Lnc1840 and lnc1459: long non-coding RNAs. Black arrows in the transcriptional networks denote direct regulation, whereas grey arrows denote indirect regulation. Question mark denotes that is uncertain if CNR forms a complex with RIN. SlCTR4, tomato *CONSTITUTIVE TRIPLE RESPONSE 4*.

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2. MIR156-TARGETED *SPL/SBPS* MODULATE GIBBERELLIN (GA) RESPONSES AND REGULATE GYNOECIUM DEVELOPMENT BY CONTROLLING BOUNDARY ESTABLISHMENT AND FLORAL MERISTEM SIZE

Abstract

Many traits of a fully developed fruit are controlled by processes that take place early in fruit development, sometimes at floral meristem level. Locule number in tomato fruits was shown to be directly controlled by meristem size. Most knowledge regarding early stages of fruit development is based on Arabidopsis dry fruits (siliques) as a model. Conversely, little is known about the control of early stages of fleshy fruit development. We showed previously that the interaction between miR156 and gibberellin (GA) pathways controls flowering time in tomato differently from what was shown in Arabidopsis. Here we use tomato as a model to study how miR156 and GA interact in the regulation of early stages of fleshy fruit development. Both the overexpression of miR156 (156OE) and increased GA levels (or loss of DELLA function in the *procera* mutant) led to the production of fruits with increased locule number, associated with upregulation of the boundary gene *GOBLET* (*GOB*) in early flower buds. Plants overexpressing miRNA-resistant versions of the miR156-targeted *SBP3* and *SBP15* (namely rSBP3 and rSBP15) produce fruits with decreased locule number. *GOB* is downregulated in flower buds of these plants. *SBP15* attenuates GA signalling, as the expression of rSBP15 in a background with increased GA levels recovers a WT-like ovary/fruit and shoot phenotype. Interestingly, floral meristem size is increased in 156OE and *procera* and decreased in rSBP3 and rSBP15 plants. We found that that miR156 and GA probably regulate gynoecium development and patterning by regulating different developmental processes: boundary establishment and the establishment of the floral meristem size. Importantly, miR156-targeted *SBP15* attenuates GA responses, while increasing levels of miR156 enhances them. Therefore, we propose that the miR156 module may control boundary establishment and meristem size via GA signalling.

Keywords: 1. MiR156; *DELLA*; Gynoecium; Fruit; *SQUAMOSA*

2.1. Introduction

Fruits are plant organs specialized in seed dispersal and present a wide morphological variety among different plant groups. Fruits can be classified according to many different morphological criteria, but one of the main dichotomies concerning fruit morphology is the distinction between fleshy and dry fruits (Giovannoni, 2004). The study of fruit development concerns the understanding of a broad number of processes that range from the transition of a vegetative to a floral meristem to the final ripening. Tomato (*Solanum lycopersicum*) has been used as a model to study ripening, which is the latest stage of fruit development. This is because tomato plants produce a commercially valuable fleshy fruit and the control of ripening determines many economically important traits (Seymour et al., 2013). However, most of the knowledge regarding genetic pathways that control early aspects of fruit development, such as

gynoecium patterning, was built up based on *Arabidopsis thaliana*, a species that produces dry fruits (siliques). Although these early stages are important to determine final characteristics of a fully developed fruit, to date few studies have focused on understanding genetic pathways that control tomato gynoecium development. Control of meristem size is a good example of how early steps of floral development defines the final traits of fruits. Tomato mutants with altered activity of the *CLAVATA-WUSCHEL* circuit show increased meristem size and produce bigger fruits with extremely high number of locules (Munos et al., 2011; Xu et al., 2015a). Importantly, natural mutations in *CLAVATA3* (*CLV3*) and *WUSCHEL* (*WUS*) were essential for tomato domestication (Muños et al., 2011; Xu et al., 2015; Zsögön et al., 2018).

We have previously shown that the microRNA156 (miR156) module controls initial steps of tomato fruit development (Silva et al., 2014). MiR156, a type of short non-coding RNA, along with its targets - members of the transcription factor family termed *SQUAMOSA PROMOTER BINDING PROTEIN–LIKE* (*SPL* or *SBP-box*) - defines the evolutionary conserved age-dependent floral pathway (Morea et al., 2016). Previous works have shown that the downregulation of miR156-targeted *SBP/SPL* in plants overexpressing miR156 (156OE) led to abnormal gynoecium and fruit development in Arabidopis and tomato (Silva et al., 2014; Xing et al., 2013). In tomato, 156OE gynoecia were formed by extra carpels and ectopic structures, and fruit showed undetermined growth and increased locule number (Silva et al., 2014). The overexpression of miR156 in *Arabidopsis* only affect gynoecium size, but no major structural alterations were reported (Xing et al., 2013). Thus, similar pathways seem to differently control the initial steps of fruit development in Arabidopsis and fleshy fruit species, such as tomato.

The phytohormone gibberellin (GA) has a complex relationship with miR156 pathway in the control of flowering time. In *Arabidopsis thaliana*, GA promotes flowering under non-inductive conditions via degradation of DELLA proteins, a group of GRAS transcription factors that repress GA signaling (Hauvermale et al., 2012). MiR156-targeted *SPL9* induces flowering by inducing *MIR172b* and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*). DELLA proteins form dimers with *SPL9* and interfere with its flowering inducing activity. Increased GA levels promote flowering by triggering DELLA degradation, thus releasing inhibition on *SPL9* (Yu et al., 2012). However, flower development is induced by the activation of *APETALA1* (*AP1*) by both *SPL9* and DELLA proteins (Yamaguchi et al., 2014). Interestingly, increased GA levels delays flowering in tomato(G. F. F. Silva et al., 2019). Loss of function of the tomato DELLA *PROCERA* (*PRO*) increases time to flowering. The mechanistic relationship between GA and miR156 still remains to be dissected in plants that negatively

respond to GA in terms of flowering time. Nevertheless, we recently showed that the *SPL9* ortholog in tomato *SBP15* does not form dimer with *PRO* (Silva et al., 2019).

Besides controlling flowering time, GA pathway controls some early aspects of reproductive development. *DELLA* was shown to control *Arabidopsis* inflorescence meristem size by regulating cell cycle in the rib meristem via *KIP-RELATED PROTEIN2* (*KRP2*). DELLA-mediated control of meristem size is also conserved in barley (Serrano-Mislata et al., 2017). GA was also shown to control style length in Arabidopsis, as loss of *DELLA* function has led to the production of gynoecia with increased style length (Fuentes et al., 2012). Concerning initial steps of gynoecium development, GA also controls ovule number by participating in ovule primordia formation. DELLA loss of function mutants produce ovaries with less ovules, whereas expression of the dominant DELLA version *rga*Δ17 with a placenta-specific promoter increases the number of ovules produced in Arabidopsis (Gomez et al., 2018). DELLA also control ovule number in *Brassica napus* and tomato (Gomez et al., 2018).

Boundaries are established and maintained during many processes in plant development (Wang et al., 2016). They are normally characterized by a region of low cell division rate and can determine the separation between an emerging organ primordium and a meristem or two or more emerging organ primordia, for example (Wang et al., 2016). Increased number of carpel leaves initiated to form a gynoecium may result from the establishment of more boundaries in the floral meristem. Indeed, the production of gynoecia with extra carpels have been reported in tomato plants that show ectopic expression of genes related to boundary establishment and maintenance (Berger et al., 2009; Hendelman et al., 2013) A subgroup of NAC family (NAM/ATAF/CUC) transcription factors named *NAM/CUC3* clade was described as boundary-defining factors in meristem and organ development. This group includes Arabidopsis *CUP-SHAPED COTYLEDON1*, *2* and *3* (*CUC1*, *CUC2* and *CUC3*) and the tomato homolog *GOBLET* (*GOB*). (Aida, et al., 1999; Berger et al., 2009; Hasson et al., 2011; Ishidaet al., 2000; Laufs, et al., 2004; Peaucelle, et al., 2007; Rubio-Somoza et al., 2014; Sieber, et al., 2007; Takada et al., 2001; Vialette-Guiraud et al., 2016). Most boundary related *NAM* transcription factors are post-transcriptionally regulated by miR164. This miRNA was shown to restrict *CUC2* and *GOB* expression to the boundary region in many developmental contexts (Berger et al., 2009; Laufs et al., 2004; Peaucelle et al., 2007; Vialette-Guiraud et al., 2016). Expression of boundary related *NAM* alleles resistant to miR164 regulation leads to gynoecia developmental defects, such as extra carpels in tomato and incompletely fused carpels in Arabidopsis (Berger et al., 2009; Peaucelle et al., 2007).

Loss of *PRO* function in *pro* mutants produce indeterminate fruit-like structures growing from the style end of the fruit, resembling 156OE phenotype (Carrera et al., 2012). We

hypothesize that miR156 targets and GA may be regulating early gynoecium development through common mechanisms. Considering the lack of information on the interaction between miR156 and GA in tomato and the resemblance between 156OE and *pro* phenotypes, here we have analyzed the relationship between miR156 and GA pathways in the control of tomato gynoecium development.

2.2. Material and Methods

2.2.1. Plant material and growth conditions

Tomato (*Solanum lycopersicum*) cv. Micro-Tom (MT) was used as wild type. All transgenic and mutant plants were in MT background. 156OE (Silva et al., 2014), *pro* (Bassel et al., 2008) and GA20oxOE (Garcia-Hurtado et al., 2012) were described previously. *Gob-4d* and *gob-3* alleles were introgressed in Micro-Tom from the M82 background previously described (Berger et al., 2009). Genetic transformation of rSBP3 and rSBP15 are described in a further section. Plants were grown as described by Lombardi-Crestana et al. (2012). Tissues for RT-qPCR were collected according to the stages described by Brukhin et al., (2003). For closed flower buds (CB) at stages 6 to 8 were collected. For pre-anthesis gynoecia, stage 10 flower buds were dissected and gynoecia were collected. For molecular characterization of rSBP3 and rSBP15 lines, terminal leaflet of third leaf were collected from 30-day-old plants.

2.2.2. Crossings

For generation of double transgenic and double mutant-transgenic plants, pollen was collected in microcentrifuge tubes and pollinated in the stigma of late pre-anthesis gynoecia. All plants were evaluated in the F1 generation except for 156OE;pro. This double transgenic-mutant was evaluated in the F2 generation, when around ¼ of the plants were homozygous for the recessive allele *pro*.

2.2.3. Locule number measurements

Fruits were cut in transverse sections to characterize number of locules. Each genotype was characterized by the percentage of the fruits that produced specific number of locules. The average of fruit number evaluated for each genotype was 200 fruits per genotype.

2.2.4. Generation of expression vectors and plant transformation

Total RNA was extracted from tomato leaves with Trizol reagent (ThermoFisher Scientific), treated with Turbo DNAse (ThermoFisher Scientific) and cDNA was synthesized using ImpromII Reverse Transcriptase (Promega). Fragments for constructs were amplified from cDNA. rSBP3 was amplified and cloned in pENTR D-TOPO (ThermoFisher Scientific). UTR regions were excluded to eliminate the miR156 binding site, as this site is located in *SBP3* 3'UTR region. For rSBP15 we used PCR overlap to produce synonymous mutations in the miR156 binding site, which is located in the CDS. Two fragments were amplified and combined using overlapping PCR. Both PCR products were purified from the gel with QIAquick Gel Extraction Kit (Qiagen) and used as a template for overlapping PCR. This product was also cloned in pENTR D-TOPO. Cloned fragments were then recombined into pk7WG2.0 (Gateway System) using LR Clonase (ThermoFisher Scientific). Plants were transformed as described (Silva et al., 2014).

For Yeast Two Hybrid assays (Y2H), *GOB* (Solyc07g062840) ORF was cloned from cDNA into pENTR D-TOPO and recombined into pDEST22 and pDEST32 (ThermoFischer Scientific). *PRO*, *SBP3* and *SBP15* Y2H constructs were described by Silva et al. (2019).

2.2.5. RT-qPCR analysis

Total RNA was extracted from leaflets, closed flower buds and developing gynoecia, treated with DNAse and reverse-transcribed to generate first-strand cDNA, as described above. PCR reactions were performed using GoTaq qPCR Master Mix (Promega) and analyzed in a Step-OnePlus real-time PCR system (Applied Biosystems). Tomato *TUBULIN* (Solyc04g081490) was used as internal control. Three technical replicates were analyzed for three biological samples (each comprising 15 closed buds, 15 gynoecia or 5 leaflets), together with template-free reactions as negative controls. The threshold cycle (CT) was determined and fold-changes for each gene was calculated using the equation $2^{-\Delta\Delta ct}$ (Livak & Schmittgen, 2001). For miRNA quantification, cDNA synthesis and qPCR reaction were performed as described (Varkonyi-Gasic et al. 2007).

2.2.6. Yeast Two-Hybrid assays

Y2H assays were performed using the pJ69-4a strain (*trp1-901; leu2-3,112; ura3-52; his3-200; gal4⊿; gal80⊿; GAL2-ADE2; lys2::GAL1-HIS3; met2::GAL7-lacZ*), which bears reporter genes for *HIS3* (histidine), *ADE2* (adenine) and *lacZ* (James et al., 1996). *GOB* (Solyc07g062840),

PRO (Solyc11g011260), *SBP3* (Solyc10g009080) and *SBP15* (Solyc10g078700) ORFs were fused to *GAL4* binding domain (BD – pDEST32) and GAL4 activation domain (AD-pDEST22). Colonies were grown on SD/-Leu/-Trp/-His and SD/- Leu/-Trp/-His/-Ade plates. Five colonies were assessed for each vector combination.

2.2.7. Confocal imaging and image analysis

Tomato reproductive apices of approximately 10 days after germination were dissected and the first floral meristem was selected in stereomicroscope for a standardized stage. Apices bearing early floral meristems with early sepals were selected, stained by modified Pseudo-Schiff with Propidium Iodide and imaged as previously described (Serrano-Mislata et al., 2015; Bencivenga et al., 2016; Serrano-Mislata et al., 2017).

For image analysis, Python scripts and Fiji macros previously described were used to segment confocal image stacks, define the position of cells within the shoot meristem, delimit meristem cells and measure L1 volume. The function of each scripts and instructions on how to perform analysis with them are described in detail in Bencivenga et al. (2016) and Serrano-Mislata et al. (2017).

2.2.8. Statistical analysis

Statistical analyses were done using SigmaPlot. Data were analysed by using two-tailed Student's T test at 5% of significance. For RT-qPCR data, statistical tests were performed considering ΔCT value of each replicate (Yung et al., 2006).

2.3. Results

2.3.1. miR156 module and GA regulate fruit locule number and control *GOBLET* expression

It was previously shown that miR156-targeted *SPL/SBPs* are critical for fruit determinacy and locule number establishment (Silva et al., 2014). Gibberellin (GA) pathway also controls fruit determinacy (Carrera et al., 2012). Tomato plants bearing *PROCERA* (*PRO/DELLA*) loss of function allele with a point mutation in the *GRAS* domain (*procera – pro*) produce fruits with fruit-like structures growing from the style end of the fruit (Figure 1A), similarly to the fruit phenotype observed for plants overexpressing the miR156 (156OE; Silva et

al., 2014). Although these structures in the fruits have already been observed in WT plants treated with GA_3, it was not shown whether altered GA signalling affects the number of locules (Carrera et al., 2012). Based on the undetermined fruit phenotypes of 156OE and *pro* mutant, our initial hypothesis was that miR156 targets and GA may act in the same pathway controlling fruit development. In order to initially address this hypothesis, we determined the number of locules in fruits produced by *pro* and plants overexpressing the *GA20ox* gene (GA20oxOE plants, which has increased levels of active GA, Garcia-Hurtado et al., 2012; Rieu et al., 2008). Fruits of both *pro* and GA20oxOE plants showed increased number of locules in comparison to MT (Figure 1C). Most MT fruits displayed two to three locules, whereas the majority of *pro* and GA20oxOE fruits showed three to four locules. The fruit phenotypes of GA-related mutant and transgenic plants was not as drastic as 156OE, which produces most fruits with four, five and six locules (Figure 1C). Although *pro* produces fruit-like structures in the style end of some fruits, none of the GA20oxOE fruits exhibited this phenotype. Around 3%, 0%, and 17% of *pro*, GA20oxOE and 156OE ovaries showed indeterminate structures, respectively (Figure S5D). To understand if decreased GA levels could reduce the number of locules in the fruits, we treated WT plants with the GA biosynthesis inhibitor Paclobutrazol (PBZ, Figure 1D). Strikingly, PBZ-treated plants produced fruits with decreased locule number. Most fruits produced by treated plants had two locules.

Number of locules in the fruits are a direct consequence of the number of carpels initiated by the floral meristem, considering that carpels fuse together to form gynoecia and each carpel originate a locule in the fruit (Seymour, et al., 2013). In tomato early flower bud primordia (0.8 to 1mm length - Brukhin et al., 2003), carpels initially emerge separately and thus fuse to form the gynoecium. The genetic regulation of these developmental stages determines many traits of a fully developed fruit, including number of locules. To initially understand the role of the miR156 pathway in early carpel development, we assessed the expression of mature miR156 and the miR156-targeted *SBP3* and *SBP15* transcription factors in two stages: 1 mm early flower buds where carpels are emerging and fusing (here referred as closed buds – CB, Figure S1 A) and an early gynoecium stage where ovary, style and stigma are already formed but not fully elongated (here referred as pre-anthesis gynoecium – Pre, Figure S1 B). Mature miR156, *SBP3* and *SBP15* are significantly more expressed in CB than in Pre (Figure 1B). This expression dynamic suggests that the pathway is recruited at early gynoecia developmental stages, mostly during carpel emergence and fusion.

GOB gain of function mutants (*Gob-4d*, for instance) were reported to produce ectopic carpels in the gynoecium, and the loss of function gob mutant (*gob-3*) produce fruits with fewer

locules than WT (Berger et al., 2009). Nevertheless, it remains unclear which pathways regulate the activity of this gene in tomato fruit development, although it is already known that GOB and some of its homologs (*CUC1* and *CUC2*) are regulated by the miR164 (Berger et al., 2009; Blein et al., 2008; Laufs et al., 2004). In fact, we have shown that overexpression of miR164 in tomato reduces locule number (Silva et al., 2014). *GOB* is highly expressed in CB in comparison to Pre, when carpels are already fused in the gynoecium (Figure 1B). This suggests that this gene is required for boundary establishment during early floral whorl development.

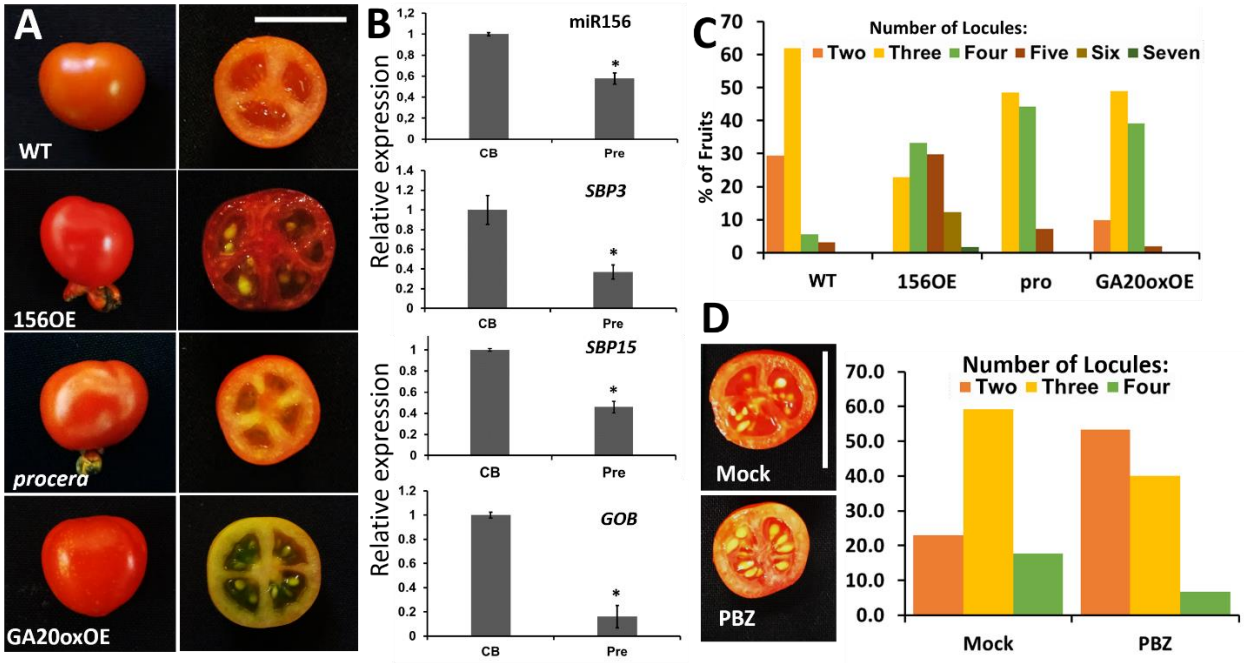

Figure 1. Phenotype of 156OE and GA-related mutant/transgenic plants and expression patterns of miR156, SBP3, SBP15 and GOB. Treatment of WT plants with (A) Fruit morphology of 156OE and GA-related mutant/transgenic plants. (B) Relative expression of miR156 and genes by RT-qPCR. Tomato TUBULIN (Solyc04g081490) was used as an internal control for all experiments. qRT-PCR values are mean ± s.e. of three biological replicates. Asterisks indicate a significant difference when compared with reference sample according to Student's t test (two tailed; *p<0.05). Bar = 2cm. (C) Percentage of fruits producing different number of locules in each genotype. (n =150 fruits). (D) Representative fruits of plants watered with 10^{-6} M of Paclobutrazol and ethanol (mock) for 20 days post germination, and percentage of fruits producing different number of locules in each treatment (n= 100 fruits). GA, gibberellin. pro, procera. CB, closed buds; pre, pre-anthesis gynoecium. PBZ, paclobutrazol.

To better understand how increased *GOB* activity affects gynoecium development we characterized ovary and fruit morphology of *GOB* gain of function heterozygous mutant (*Gob-4d* – allele with a point mutation in the miR164 binding site). Besides affecting gynoecium and fruit shape, ectopic expression of *GOB* increases number of locules in the fruit (Figure 2A and B). Most fruits produce four to six locules (Figure 2B), and the ovaries have partially fused carpels, with unfused style and stigma in the early stages of gynoecium development (Figure 2C, arrow). To shed light on the possible connection between the boundary gene *GOB* and the miR156 and GA pathways we assessed *GOB* expression in closed flower buds (CBs) of 156OE and *pro.*

Consistent with 156OE and *pro* locule number phenotypes, *GOB* expression is induced in CB of these genotypes in comparison to MT (Figure 2D). This data suggests that *SBP* transcription factors and GA may regulate boundary establishment during carpel morphogenesis partially via *GOB*. To better clarify this idea, we are generating double mutants between miR156 module and *Gob-4d*, and we are treating *Gob-4d* mutants with paclobutrazol (PAC), a GA inhibitor (Lee et al., 1985).

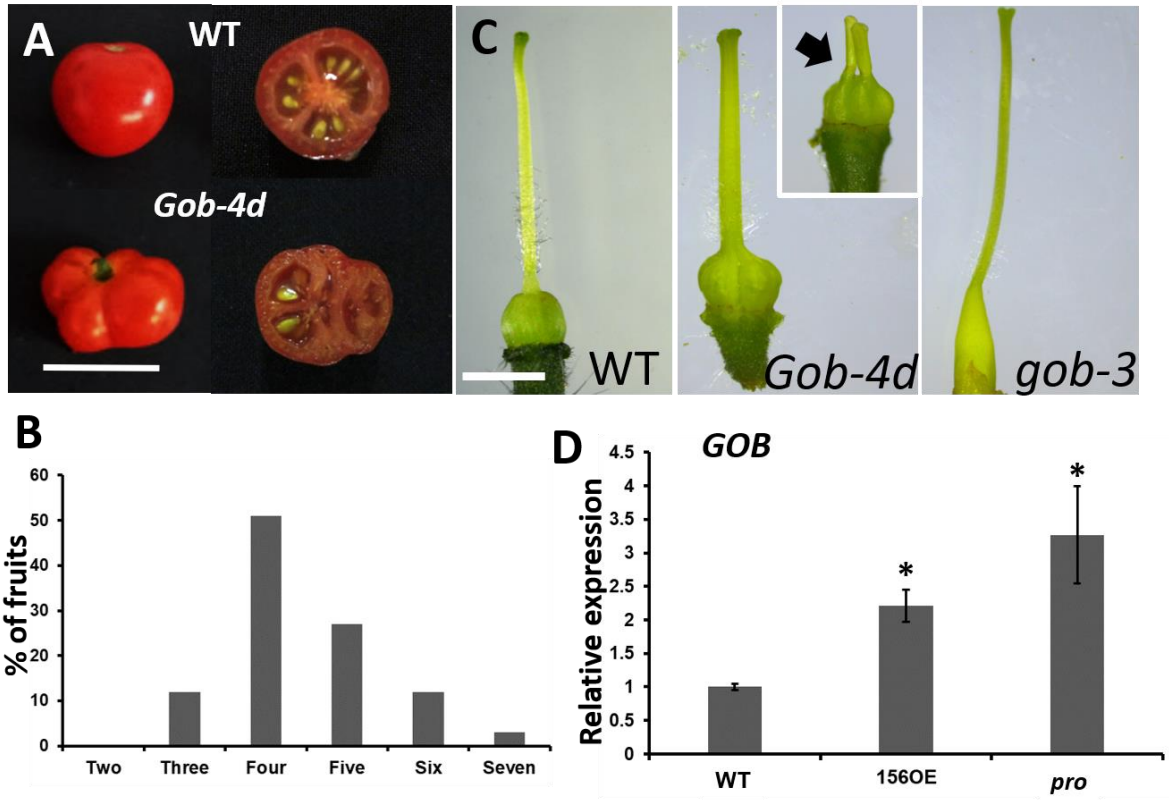

Figure 2. GOB mutant phenotypes and GOB expression in CBs of 156OE and pro plants. (A) WT fruits and fruit phenotype of GOB gain of function mutant Gob-4d. Bar = 2cm. (B) Percentage of fruits with different number of locules in Gob-4d. (n = 150 Fruits) (C) Ovaries of WT, Gob-4d and gob-3 (loss of function mutant - Berger et al., 2009). Bar= 2mm. Arrow points unfused style and stigma in a pre-anthesis gynoecium. (D) GOB transcript accumulation in 156OE and pro CBs. Tomato TUBULIN (Solyc04g081490) was used as an internal control for all experiments. qRT-PCR values are mean ± s.e. of three biological replicates. Asterisks indicate a significant difference when compared with reference sample according to Student's t test (two tailed; *$p<0.05$). CBs, closed buds.

2.3.2. Distinct miR156-targeted *SBP/SPL* transcription factors regulate locule number and *GOBLET* expression

Arabidopsis miR156-targeted *SPL*s and the non-targeted *SPL8* have partly redundant roles in the regulation of gynoecium development (Xing et al., 2013). The overexpression of miR156 leads to a reduction in gynoecium size without major changes in organ structure, and *spl8* mutant shows no obvious gynoecium phenotype. However, *spl8*;p35S:MIR156b plants produce

gynoecia with altered shape, major structural changes in septum and absence of transmitting tract (Xing et al., 2013). MiR156-targeted *SPL/SBP* downregulation in tomato 156OE is enough to produce gynoecia with several ectopic carpels, suggesting that non-targeted *SBPs* may not have major roles in gynoecium development (Silva et al., 2014). The miR156-target *SPLs* are subdivided into two major groups based on sequence, size and gene structure. The group that includes *SPL9* and *SPL15* is represented by larger proteins, due to the presence of a C-terminus domain not found in the other miR156-targeted *SPL*s. The transcripts of this group bear a miR156 binding site in the CDS. The group that includes *SPL3* code for smaller proteins, without the C-terminus domain. These small *SPL*s are constituted basically by the *SPL* domain, and the miR156 binding site is located in the 3'UTR region of the transcripts of this group (Birkenbihl et al., 2005; Cardon et al., 1999; Xing et al., 2010). These two major groups also have divergent roles. Although both groups have specific roles in floral induction and flower development, the *SPL9/15* group also control vegetative traits such as leaf morphology and leaf initiation rate (Hyun et al., 2016; Jung et al., 2012; Rubio-Somoza et al., 2014; Schwarz, et al., 2008; Usami et al., 2009; Wang et al., 2009; Wu et al., 2010; Wu & Poethig, 2006).

The role of specific miR156-targeted *SPLs* in carpel development remains unknown. Analysing the functional roles of small and large miR156-targeted *SPL/SBP* is fundamental to understand how the roles of different clades of miR156 targets diverge in the control of carpel development. Toward this end, we generated independent lines of MT plants overexpressing miR156-resistant versions of *SBP3* and *SBP15* (abbreviated as rSBP3 and rSBP15, respectively). Figure 3D shows schematic representations of *SBP3* and *SBP15* transcripts and mutations inserted by PCR in the miR156 binding site of *SBP15*. r*SBP3* was generated by simply not cloning the 3'UTR, where miR156 binding site is located (Figure 3D), into the construct.

Strikingly, plants from the rSBP3 and *SBP15* independent lines produced fruits with reduced number of locules (Figures 3A, 3B, S2 and S3). Most fruits of these plants produce two locules, and some produce three locules (Figure 3A and 3B). Fruit shape was also changed. In all lines of both transgenics the fruits are more elongated and have a constriction in the style end (Figure 3A). Interestingly, *SBP3* and *SBP15* seem to have different roles in plant architecture. rSBP3 plants architecture is similar to MT, whereas rSBP15 lines produce semi-dwarf plants (Figures S2D and S3D).

For further analysis we chose the two lines of each transgenic that displayed the highest levels of transgene overexpression. To better understand whether miR156 pathway controls number of locules in the ovary/fruit we assessed *GOB* expression in two independent lines of each transgenic plant. *GOB* expression was downregulated in the two lines of rSBP3 and rSBP15

(Figure 3C). Interestingly, small and large tomato *SBP* transcription factors seem to have conserved roles in the transcriptional control of *GOB* levels in the gynoecium.

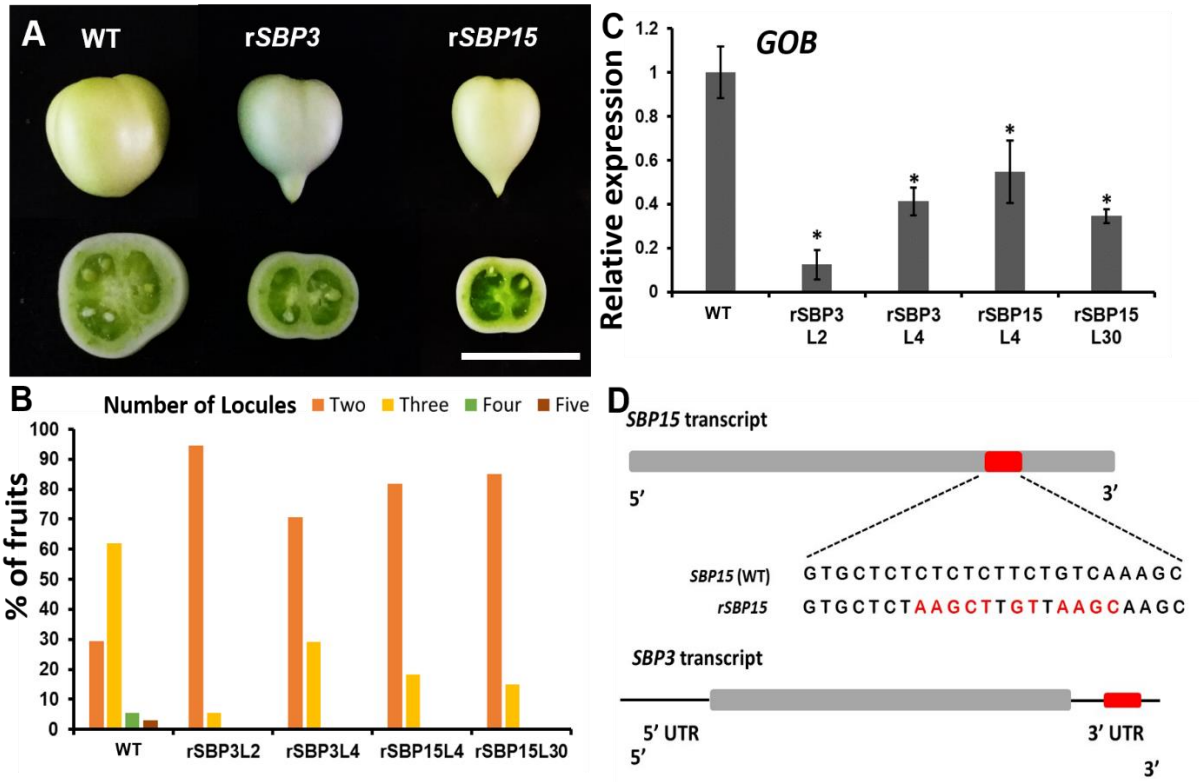

Figure 3. Overexpression of miR156-targeted SBP3 and SBP15 reduce fruit locule number and represses GOB expression in CBs. (A) WT fruits and fruit phenotype of rSBP3 and rSBP15 plants. Bar = 2cm. (B) Percentage of fruits producing different number of locules in two independent lines of rSBP3 and rSBP15 (rSBP3 lines #2 and #4; rSBP15 lines #4 and #30) . (n = 150 fruits) (C) GOB transcript accumulation in two independent lines of rSBP3 and rSBP15. Tomato TUBULIN (Solyc04g081490) was used as an internal control for all experiments. qRT-PCR values are mean ± s.e. of three biological replicates. Asterisks indicate a significant difference when compared with reference sample according to Student's t test (two tailed; *p<0.05). (D) Schematic representation of SBP3 and SBP15 transcripts. Grey boxes represent CDS, red boxes represent miR156 binding site, black lines represent 5' and 3'UTR regions. Sequence shows mutated bases (in red) in the miR156 binding site to produce rSBP15. rSBP3 was obtained by not cloning the 3'UTR region.

2.3.3. miR156 and GA synergistically regulate gynoecium development

Although GA and miR156 pathways are capable of regulating *GOB* levels in CBs, it remains unclear whether they act in a common pathway upstream of *GOB* or they are part of more than one pathway that converge into *GOB* transcriptional regulation. Assessing gynoecium and fruit morphology of plants with increased GA levels or decreased *DELLA* activity that also has high levels of miR156 is a useful tool to initially address this question. Thus, we generated 156OE;GA20oxOE and 156OE;*pro* double transgenic and double transgenic mutant plants, respectively, and a synergic effect was observed. 156OE; GA20oxOE and 156OE;*pro* plants are taller than the respective parental plants, with modified vegetative architecture (Figure S4A and

B) and produced extremely amorphous and undetermined fruits, with no definition of locules (Figure S4C). These fruits are composed by an amorphous pericarp mass and indeterminate fruit-like structures (Figure 4). Gynoecia are mostly formed by multiple ectopic unfused carpels and ectopic structures (Figure 4D shows the intact carpels, and, as shown in Figure S5, some carpels were dissected/removed to evidence the ectopic structures). Figure S5 shows 156OE; *pro* gynoecia in different developmental stages, emphasizing the ectopic structures that grow from the ovaries. Undetermined ectopic structures could be detected in gynoecia of 100% of 156OE; *pro* and 156OE; GA20oxOE ovaries from the first inflorescence (Figure S5D). Considering the severity of the modifications and the frequency of ectopic structures growing from the ovary in the double mutants, it is reasonable to assume that these phenotypes exceed possible additive effects, thus characterizing the phenotype as a result of a synergistic effect.

Some studies reported synergy resulting from disruption of two steps of a pathway that converge in a node. For example, Arabidopsis auxin efflux protein *PIN-FORMED1* (*PIN1*) mutant *pin1* form leaves and other lateral organs with slight defects. The same was observed in double mutants for auxin biosynthetic enzymes *YUCCA1* and *YUCCA4* (*YUC1* and *YUC4*). Both *PIN* and *YUC* are multigene families, and other member may compensate for the aforementioned mutations. But *pin1;yuc1;yuc4* triple mutants, which show partially disruptions in both auxin efflux and biosynthesis, fail to form normal leaves (Cheng, et al., 2007).

If control of locule number occurs via controlling boundary initiation, it is possible that miR156 and GA pathways redundantly control *GOB* levels. However, *GOB* transcript accumulation levels in 156OE;*pro* CBs is similar to levels observed in *pro* (Figure 4C), suggesting that transcriptional control of this gene may not account for the synergic effect observed. Functional and phylogenetic studies showed that *Arabidopsis NAM/CUC3* group is composed by the *CUP-SHAPED COTYLEDON 1*,*2* and *3* (*CUC1, CUC2* and *CUC3)* and actually subdivided into 2 subgroups: *NAM* and *CUC3*. This division was based in *NAC* domain amino acid sequences and presence/absence of miR164 binding site. *CUC1* and *CUC2* are grouped in the *NAM* subgroup whereas *CUC3* is grouped in the *CUC3* clade. *GOB* belongs to the *NAM* subgroup, and no tomato protein groups with *CUC3*. Although *CUC3* is not a miR164 target *CUC1*, *2* and *3* have partly redundant functions the control of leaf development (Blein et al., 2008; Hasson et al., 2011). Recent data suggest *NAM* plus *CUC3* was a single miR164-regulated lineage in the gymnosperms and diversified in *NAM* and *CUC3* via genome duplication. Loss of miR164 binding site may have occurred later (Vialette-Guiraud et al., 2011).

A post-translational mechanism controlling heteroblasty in *Arabidopsis* and *Cardamine hirsuta* was proposed to control leaf complexity by controlling dimerization of the boundary genes

CUC2 and *CUC3* (Rubio-Somoza et al., 2014). In this mechanism, titration of *CUC2-CUC2* and *CUC2-CUC3* dimmers control leaf complexity, and *TCP4* binds to *CUC2* and prevent the formation of these *CUC* dimmers. High levels of *TCP4* during leaf organogenesis decreases the amount of CUC dimmers and leads to the production of simpler leaves (Rubio-Somoza et al., 2014). It is unknow whether such mechanism may be preserved in other organs.

To initially address this question, we tested by Yeast Two Hybrid (Y2H) whether *PRO*, *SBP3* and *SBP15* form dimers with *GOB*. Phylogenetic analysis suggest that *GOB* is the only *CUC2* and *CUC3* ortholog in tomato, thus this mechanism may operate in tomato organogenesis through the control of abundance of *GOB-GOB* dimers. In order to initially address whether *GOB* proteins can interact and form homodimers, we tested this interaction via Y2H assays (Figure 3D). *GOB* is capable of auto activating the system when fused to *GAL4* binding domain. However, supplementation with 10mM of the histidine biosynthesis inhibitor 3-amino-1,2,4-triazole (3-AT) in the culture media was sufficient to prevent growth of colonies caused by auto activation (AD-, BD-GOB), whereas 3-AT did not inhibit the growth of colonies due to *GOB-GOB* interaction (AD-*GOB*, BD-*GOB*). This data suggests that *GOB* can form homodimers. Considering this data, it is possible that proteins that bind *GOB* are capable of disrupting *GOB-GOB* dimers by competing with other *GOB* proteins, as shown in *Arabidopsis* for *CUC2* and *CUC3*. In order to understand whether miR156 targets or *PRO* could bind directly to *GOB* we performed more Y2H assays. *SBP3* and *SBP15* do not interact with *GOB* directly. However, a strong interaction between *GOB* and *PRO* was detected, considering that yeasts transformed with AD-*GOB* and BD-*PRO* grew even with 10mM 3-AT. This result suggests that miR156 targets can control *GOB* at transcriptional levels, while *PRO* may control *GOB* activity both at transcriptional and post transcriptional levels (Figures 2 and 3). Our hypothesis is that *GOB-GOB* homodimer promotes boundary initiation, as it happens with its *Arabidopsis* homologs, and *PRO* may be capable of binding *GOB* and control the number of *GOB-GOB* dimers available in the cell. Although more experiments are still required to test this hypothesis, the phenotypes of our plants corroborate the existence of this network.

GOB is post transcriptionally regulated by miR164 (Berger et al., 2009). Thus, *SBP* and GA pathways may regulate *GOB* transcript levels via controlling miR164 expression. To clarify this, we assessed mature miR164 accumulation in CBs of 156OE, rSBP3, rSBP15 plants (Figure S6). Consistent with this hypothesis, miR164 levels were downregulated in 156OE CBs. However, rSBP3 and rSBP15 also showed downregulated levels of miR164. Thus, *GOB* downregulation in these genotypes are not explained by miR164 accumulation. This data suggests that other miR156-targeted SPL/SPBs might be involved in the regulation of *GOB* levels via

miR164. We also assessed miR164 expression in 156OE;*pro* (Figure S6), which showed unchanged mature miR164 levels in comparison to WT. Thus, PROCERA appears to regulate *GOB* levels independently of miR164 regulation.

Tomato recessive mutants *clausa* (*clau*) were reported to produce indeterminate fruits with fruit-like structures growing from the style end, resembling 156OE and *pro* phenotypes (Avivi et al., 2000) (Figure S7). This mutation was later cloned and better characterized functionally. *CLAU* is a Solanaceae specific *MYB* transcription factor that attenuates cytokinin signalling and represses *GOB* expression in tomato leaves (Bar et al., 2015; Bar et al., 2016). *CLAU* deregulation might be correlated with the indeterminate growth and *GOB* transcriptional regulation in 156OE and *pro* plants (Figure S7). The expression of *CLAU* in rSBP3 and rSBP15 CBs was not significantly different from WT. *CLAU* transcriptional levels may be regulated by miR156-targeted *SPL/SBPs* other than *SBP3* and *SBP15*.

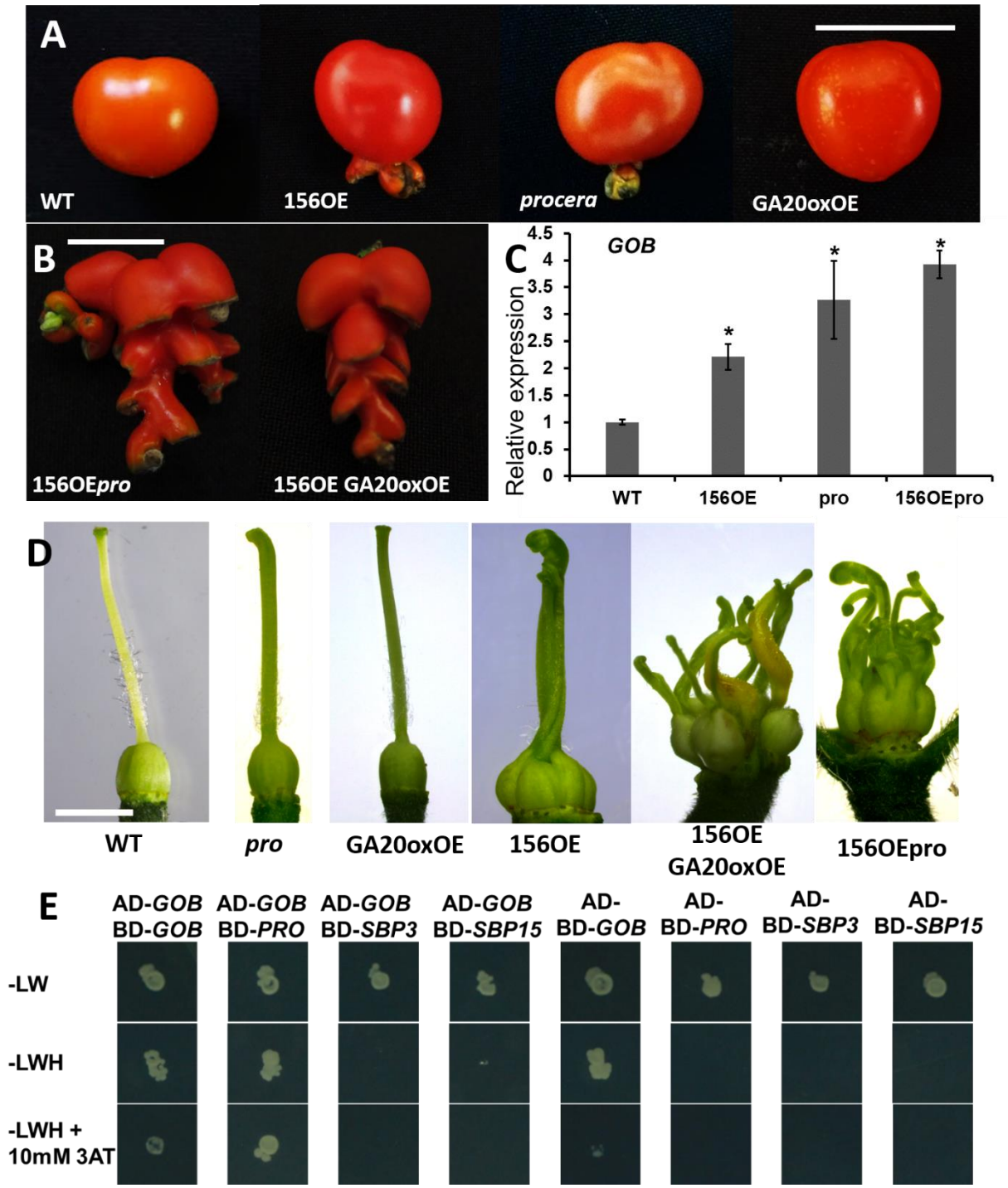

Figure 4. miR156 and GA pathways synergistically regulate gynoecium and fruit development. (A) and (B) Fruit morphologies of 156OE, pro and GA20oxOE plants compared to 156OE;pro and 156OE;Ga20oxOE. Bars = 2cm. (C) GOB expression in 156OE;pro in comparison to GOB levels in WT, 156OE and pro.Tomato TUBULIN (Solyc04g081490) was used as an internal control for all experiments. qRT-PCR values are mean ± s.e. of three biological replicates. Asterisks indicate a significant difference when compared with reference sample according to Student's t test (two tailed; *$p<0.05$). (D) Gynoecium phenotypes of mutants, transgenic and double mutant-transgenic plants. Bar = 2mm. (E) Two-hybrid system (Y2H) assays. GOB was fused to both GAL4 binding domain (BD) and GAL4 activation domain (AD). SBP15, SBP3 and PRO were fused toGAL4 BD. Colonies were examined on SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade plates. Five clones were analysed for each interaction, and one is shown.

2.3.4. *SBP15* attenuates GA responses

Increased plant height is a phenotype related to increased GA signalling in many plant species (Daviere & Achard, 2013). *DELLA* was shown to control plant height by blocking the activity of Class I *TCP* transcription factors in *Arabidopsis* shoot apex (Davière et al., 2014). The extreme plant height and fruit phenotypes observed in 156OE;*pro* and 156OE;GA20oxOE (Figures 4 and S6) are consistent with the hypothesis that miR156-targeted *SPL/SBPs* may modulate GA responses. If *SPL/SBP* downregulation in 156OE is capable of increasing GA responses, upregulation of specific *SPL*s may be capable of attenuating GA response or decrease GA sensitivity. To test this hypothesis, we expressed rSBP3 and rSBP15 in backgrounds with plants showing increased GA biosynthesis. We generated rSBP3;GA20oxOE and rSBP15;GA20oxOE double transgenic plants. Strikingly, high *SBP15* levels attenuate GA responses. Instead of typically tall plants, rSBP15*;* GA20oxOE are also semi-dwarf like rSBP15 plants (Figure 5A). A WT-like phenotype in terms of locule number distribution was rescued in the fruits (Figure 5C). This phenotype rescue was not observed in rSBP3;GA20oxOE plants. These plants display phenotypes resembling GA20oxOE plants both in terms of plant height and fruit locule number (Figure 5). This data points to the divergent regulatory roles of large and small *SPL/SBP*s in the regulation of gynoecium development. Increased levels of both transcription factors are capable of downregulating *GOB* and decrease number of carpels initiated in the floral meristem. However only *SBP15* is capable of attenuating GA responses. It is possible that *SBP15* regulates many aspects of plant development via modulation of GA responses.

Our data indicates that a point of interaction between miR156 and GA pathways is the control of GA responses by *SBP15*. To understand if GA pathway have any type of role in the transcriptional control of miR156 targets, we assessed the expression of four miR156 targets in *pro* CBs: *SBP2*, *SBP3*, *SBP10* and *SBP15* (Figure S8). Interestingly, *SBP15* is the only gene that was considerably upregulated in *pro* (Figure S8). Apparently, there is a feedback loop in which *SBP15* negatively regulates GA responses while *PRO* represses *SBP15*. It may be a mechanism related to the fine-tuning of GA responses in plant cells. To better test this hypothesis, we are currently treating WT, 156OE, and rSBP15 plants with paclobutrazol (PAC), an inhibitor of GA biosynthesis (Jung et al., 2012).

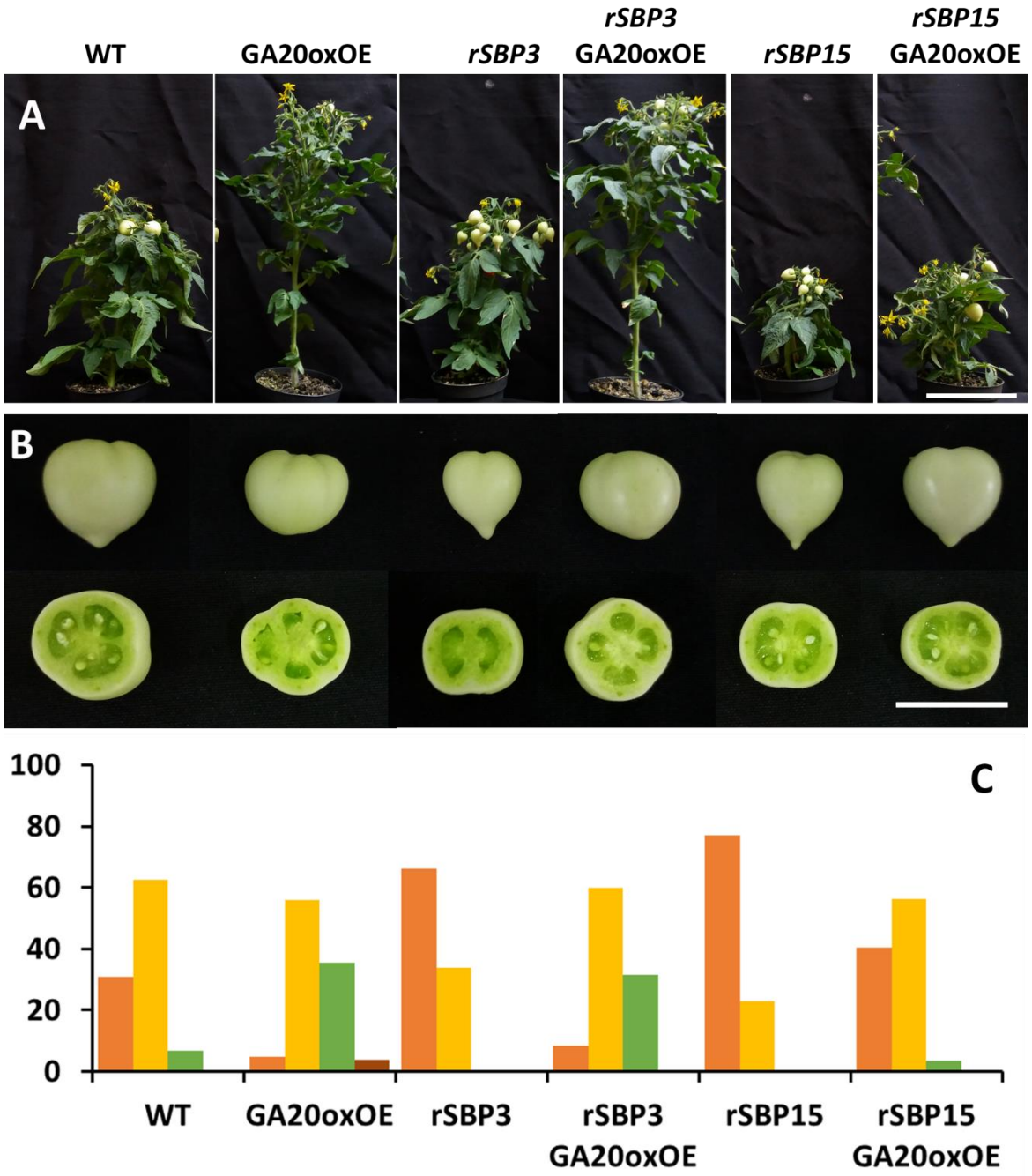

Figure 5. High SBP15 levels attenuate GA responses in tomato. Representative shoot (A) and fruits (B) of WT, GA20oxOE, rSBP3, rSBP3;GA20oxOE, rSBP15, and rSBP15;GA20oxOE. GA-induced shoot elongation is completely inhibited in rSBP15;GA20oxOE. Bars = 7cm. (C) Percentage of fruits producing different number of locules in WT, GA20oxOE, rSBP3, rSBP3;GA20oxOE, rSBP15,and rSBP15;GA20oxOE. (n= 100 fruits).

2.3.5. Among the miR156-targeted *SPL/SBPs*, *SBP15* has a major role in controlling gynoecium development

Higher levels of *SBP3* and *SBP15* downregulate *GOB* transcript levels in CBs, although they diverge functionally concerning their role in modulating GA responses. Thus, these genes seem to have partly overlapping functions in regulating gynoecium development, but they are not

completely redundant. To further investigate specific roles of each of these transcription factors among other miR156-targeted *SPL/SBP*s we generated 156OE;rSBP3 and 156OE;rSBP15 double transgenic plants (Figure 6 and S9). These plants present downregulated levels of all miR156-targeted *SPL/SBP*s (Silva et al., 2014) except for one specific (either *SBP3* or *SBP15*). While 156OE;rSBP15 rescued WT-like gynoecium morphology and fruit locule number,156OE;rSBP3 showed only a partial phenotype recovery. Interestingly, 156OE;rSBP3 produced fruits with less locules than 156OE but did not recover a WT-like frequency. Furthermore, ovary shape was not totally recovered. Part of the rSBP3;156OE gynoecium show incomplete fused style and stigma (Figure 6, arrow). *SBP15* is probably a major miR156 target controlling gynoecium development and it interacts with more pathways in this regulation. Moreover, plant shoot architecture was more modified by the expression of rSBP15 in a background overexpressing miR156. Instead of the 156OE-like bushy and highly branched vegetative architecture (Silva et al., 2014), 156OE;rSBP15 plants show a less branched shoot architecture (Figure S9). It has been described in *Arabidopsis* that the *SBP15* orthologs *SPL9* and *SPL15* controls shoot branching (Schwarz et al., 2008). On the other hand, 156OE;rSBP3 plants did not show altered plant architecture in comparison with 156OE plants (Figure S9). We have recently obtained rSBP15*;Gob-4d* double mutant transgenic plants and we are currently assessing fruit and ovary phenotype to understand whether *GOB* downregulation by high *SBP15* levels can attenuate *Gob-4d* phenotype.

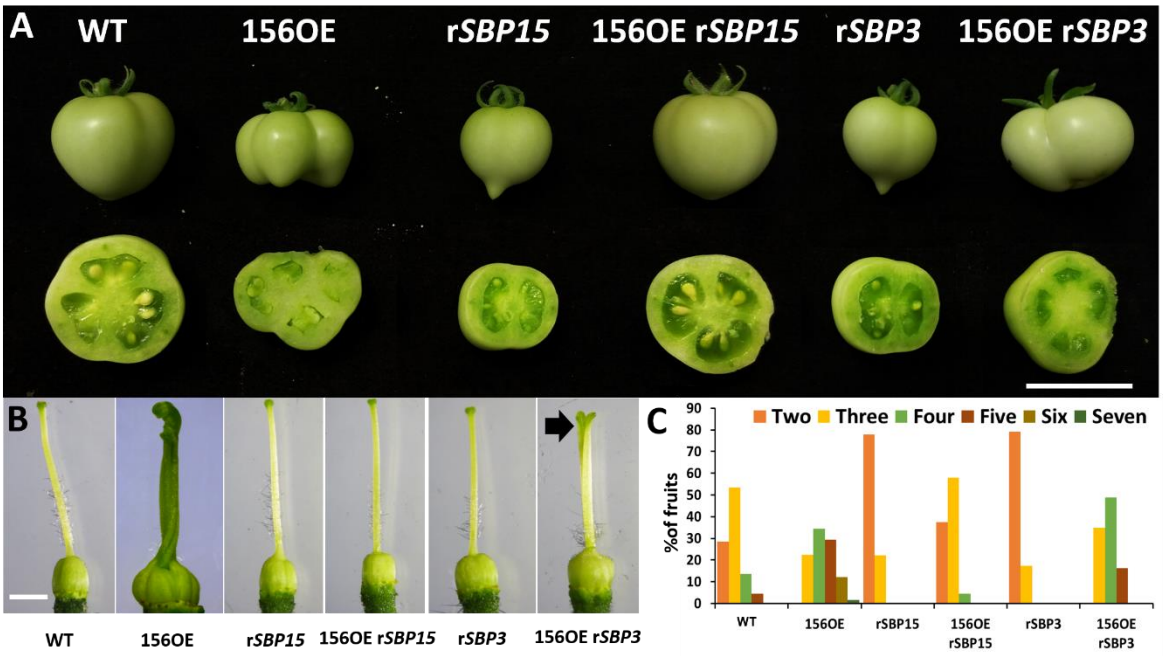

Figure 6. Expression of a miR156-resistant version of SBP15 recovers WT-like fruit phenotype in 156OE plants. (A) and (B) Representative WT, 156OE, rSBP15, 156OE;rSBP15, rSBP3 and 156OE;rSBP3 fruits and ovaries. Arrow in (B) indicates unfused region in the style and stigma. (C) Percentage of fruits producing different number of locules in each genotype. (n=80 fruits). (A) Bar = 2 cm. (B) Bar = 2mm.

2.3.6. miR156 and GA regulate floral meristem size

We showed that regulation of boundary establishment and maintenance is an important factor in the control of locule number in tomato ovary/fruit. Furthermore, regulation of meristem size was also associated with the production of flowers with increased number of whorls (Muños et al., 2011; Xu et al., 2015). As a consequence of increased number of carpels, tomato plants produce fruits with increased number of locules in genotypes where disruption of *CLAVATA-WUSCHEL* circuit lead to increased meristem size (Muños et al., 2011; Rodriguez-Leal et al., 2019; Xu et al., 2015a). To understand if higher locule number observed in genotypes associated with miR156 and GA pathways is a result of the control of meristem size, we measured floral meristem dome of *pro*, 156OE, rSBP3 and rSBP15 plants We also measured *Gob-4d* floral meristem size to understand if manipulation *GOB* activity may affect meristem size and shed light over the relationship between control of boundary establishment and control of meristem size. In order to precisely measure floral meristem size, we first established in tomato the modified Pseudo-Schiff Propidium Iodide and image analysis techniques described for Arabidopsis (Bencivenga et al., 2016; Serrano-Mislata et al., 2017) (Figure 7). After successfully establishing this approach for tomato, we used the volume of L1 layer of meristem dome as a proxy of meristem surface area. Cells considered for measurement are highlighted in red in Figure 7A. Representative raw confocal images of each genotype are shown in Figure S10. Both

pro and 156OE showed increased meristem surface area, while rSBP3 and rSBP15 showed decreased meristem area. Thus, variation in fruit locule number seems to be correlated with floral meristem size in these genotypes. Interestingly, *Gob-4d* L1 volume did not vary significantly in comparison to WT. This suggests that changes in boundary formation do not affect meristem size. MiR156 and GA pathways seem to control gynoecium development by regulating different stages of floral development. In early floral meristem development, these pathways may regulate genes associated with meristem maintenance may be regulated. In further stages, when boundary establishment determine the number of carpels initiated, *GOB* seems to be controlled by GA and miR156.

It is worth to note that meristem increasing in 156OE and *pro* and decreasing in rSBP3 and rSBP15 is also associated with variation in cell size. Moreover, 156OE and *pro* has significantly more cells in the meristems. Thus, the area of the meristems is larger both due to increased cell size and increased number of cells in the meristem. However, rSBP3 and rSBP15 meristems show smaller surface cells but does not have a significantly lower number of cells in the meristem.

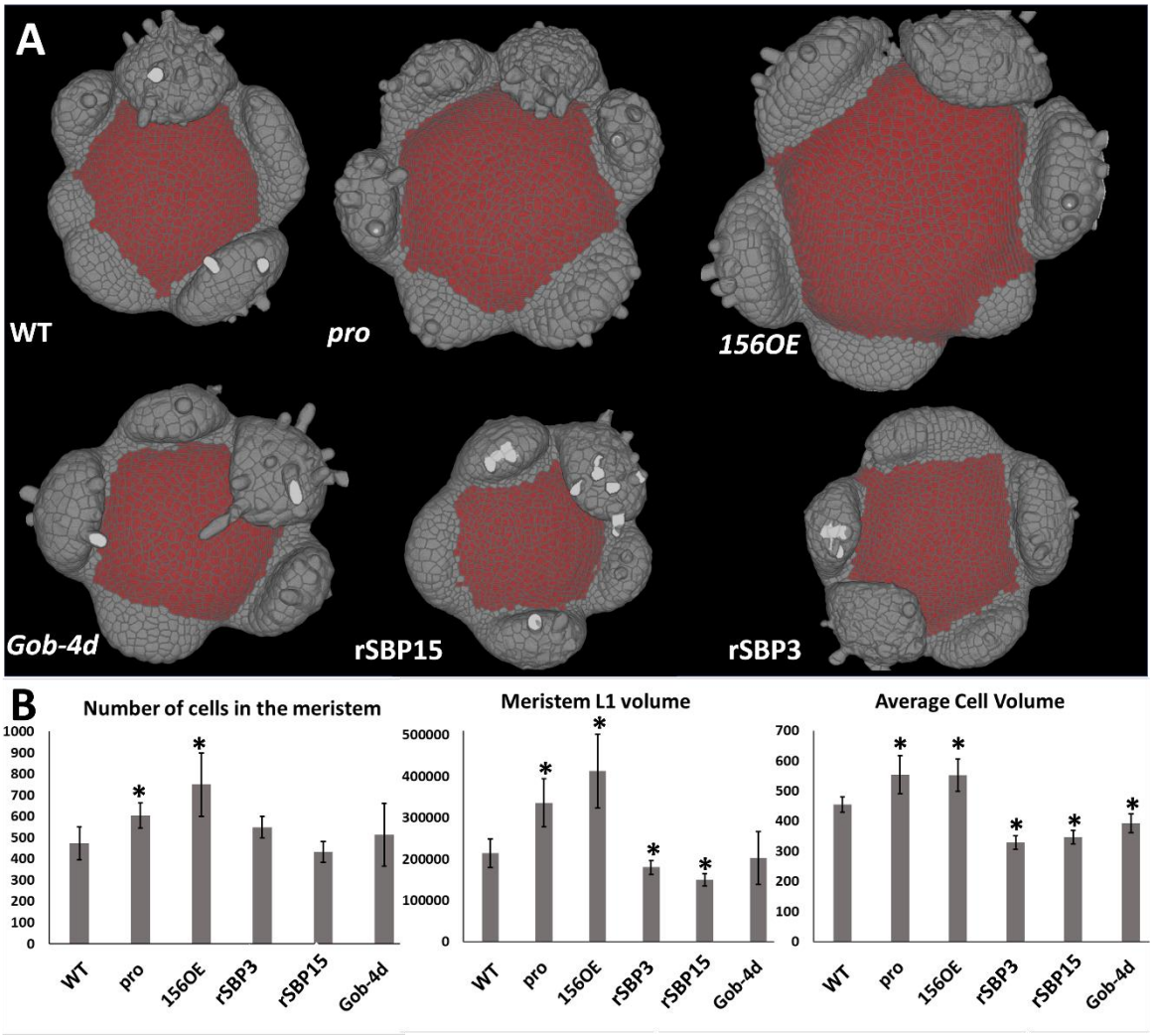

Figure 7. miR156 and GA pathways control floral meristem size in tomato. (A) 3D reconstruction of processed confocal stacks of floral meristems stained with mPS-PI. Representative floral meristems of WT, pro, 156OE, Gob-4d, rSBP15 and rSBP3 plants. Cells highlighted in red were considered for measurement. (B) Number of cells in the meristem, Meristem L1 volume and average cell volume in each genotype. Asterisks denote significant difference from control (WT) according to Student´s T test at 5% significance.

2.4. Discussion

Some aspects of the complex interaction between miR156 and GA has been relatively well characterized in the control of *Arabidopsis* flowering time. However, GA response itself is very complex and pleiotropic, and molecular mechanisms of *DELLA* action are still poorly understood. Furthermore, we know from our previous study that miR156 and GA interaction differ between tomato and Arabidopsis in a key step of flowering time control: in *Arabidopsis* GA promotes flowering by releasing DELLA-bound SPL9 proteins, whereas in tomato GA delays flowering time and DELLA does not interact with SBP15.

The overexpression of miR156 has major effects on gynoecium and fruit development, probably related to mis-regulation of specific sets of genes (Silva et al., 2014). Here we show that miR156 and GA probably regulate gynoecium development and pattern by regulating at least two

temporally separated developmental processes: boundary establishment and meristem size. Importantly, miR156-targeted *SBP15* attenuates GA responses. Thus, miR156 module may regulate many developmental processes by modulating GA responses, including the control of cell expansion.

Boundaries shape many aspects of plant body. *NAM/CUC* genes are expressed in boundary zones and the loss of their function leads to the production of adjacent fused organs or less complex organs. Also, their ectopic expression produces defects related to increased boundary establishment, such as more complex leaves and gynoecia with extra carpels (Berger et al., 2009; Hendelman et al., 2013). They were proposed to be key players in boundary establishment, although their downstream targets and related mechanisms are still poorly understood. *NAM/CUC* mutants produce gynoecium developmental defects in several species. In the *Medicago trunculata MtNAM* null mutants, gynoecia is composed by unfused carpels with exposed ovules (Cheng et al., 2012). *Arabidopsis cuc1;cuc2* double mutants show unfused septum and fewer ovules (Ishida et al., 2000). Unlike *GOB*, another miR164-targeted tomato boundary-related *NAM* gene *SlNAM2* is expressed only after carpels had already been fused, and most likely acts in boundary maintenance rather than boundary establishment. Overexpression of miR164-resistant *SlNAM2* leads to the production of extra carpels, similarly to *Gob-4d* phenotype (Hendelman et al., 2013). Two important questions regarding boundary establishment is when and how *CUC-like* genes are active in some developmental processes? In tomato, *SBP3* and *SBP15* seem to be responsible for controlling *GOB* activity during carpel development. MiR156, *SBP3*, *SBP15* and *GOB* are recruited early in CB and less expressed in Pre-anthesis gynoecia (Figure 1C). This coexpression pattern, together with ovary/fruit phenotypes of 156OE, rSBP3, rSBP15 and *Gob-4d* corroborates with the hypothesis that recruitment of miR156 module in early stages of carpel development is necessary to fine tune *GOB* levels or limiting its physical expression domains to specific regions during flower development. *GOB* downregulation in rSBP3 and rSBP15 may account for less boundary zones established during carpel development, thus leading to the emergence of less carpels from the flower meristem and production of fruits with decreased locule number. Transcriptional regulation of *NAM/CUC* genes by *SPL/SBP* genes have not been reported so far. The only relationship between miR156 targets and *NAM/CUC* was reported in a different developmental context. *Arabidopsis SPL9* was shown to repress *LATERAL SUPRESSOR* (*LAS*) expression in boundary establishment during axillary bud formation, whereas *CUC2* directly induces *LAS* expression (Tian et al., 2014).

Rubio-Somoza et al. (2014) proposed a mechanism by which *CUC2* and *CUC3* control leaf complexity, and their activity is indirectly controlled by *SQUAMOSA PROMOTER-*

BINDING PROTEIN-LIKE 9 (*SPL9*), which is a *SBP15* ortholog. This mechanism promotes temporal control of leaf complexity. *CUC2* and *CUC3* dimmers are effectors of boundary establishment, and control of *CUC2-CUC3* and *CUC2-CUC2* dimer abundance modulates boundary initiation in leaf margins and thus leaf complexity. CUC2-CUC2 dimers are titrated by *TCP4*. *TCP4* interacts with *CUC2*, preventing the formation of *CUC* homo and heterodimers. The miR156 pathway adds another level of regulation in this mechanism: as miR156 expression decreases through plant age, its targets accumulate more in the tissues. miR156-targeted *SPL9* is capable of binding to TCP4 and disrupt *TCP4-CUC2* heterodimers, thus releasing *CUC2* to form *CUC2-CUC2* and *CUC2-CUC3* dimers, which promote leaf complexity. So, when more *SBP9* is available in the cells to bind to *TCP4*, more *CUC* dimers are available to promote complexity. However, it is unknown whether such mechanism is conserved in different developmental contexts or different species. It is unlikely that this mechanism involving the homologs of *TCP4* (*LANCEOLATE – LA*), *CUC2* (*GOB*) and *SBP9* (*SBP15*) is conserved in tomato. We showed in a previous study that *LA* proteins do not interact with *SBP15* by Yeast Two Hybrid (Y2H) and Bimolecular fluorescence complementation (BiFC) assays (Silva et al., 2019). However, a similar post translational mechanism may operate in boundary establishment also in tomato

Besides regulating *GOB* transcriptional levels, *PRO* proteins interact with *GOB*. If *GOB-GOB* homodimers are necessary to transactivate downstream targets, as described for *CUC2-CUC2* and *CUC2-CUC3* dimers (Rubio-Somoza et al., 2014), it is possible that *PRO* controls boundary establishment by hijacking GOBLET proteins and preventing GOBLET homodimer formation. It is also possible that PRO interacts with GOB DNA binding domain and blocks its function, as it happens between *Arabidopsis* DELLAs and PHYTOCHROME INTERACTING FACTOR3 and 4 (De Lucas et al., 2008; Feng et al., 2008). Both situations explain how boundary formation in 156OE;*pro* and 156OE;GA20oxOE may be increased by transcriptional upregulation of *GOB* and also increased amount of available *GOB-GOB* dimers. The upregulation of *GOB* in *pro* may also be related to the increased amount of *GOB-GOB*, considering that in *CUC2-CUC2* homodimers are capable of inducing *CUC3* expression in *Arabidopsis* leaf development (Rubio-Somoza et al., 2014). The point mutation in VHIID domain in *pro* may have reduced *PRO* ability to interact with other proteins, thus releasing more *GOB* proteins to form homodimers and establish more boundaries during carpel emergence. *Gob-4d* displays diffuse expression of *GOB* in multiple points of leaf primordia instead of determining sharp boundaries of *GOB* expression with adjacent *GOB*-less region (Berger et al., 2009). Therefore, instead of producing leaves with more leaflets, these leaflets fail to separate and form leaf lobes. This may be the case of carpel leaves in 156OE;*pro* and 156OE ;GA20oxOE plants, in which aberrant

fruits do not have defined locules (Figure 4 and S4). The induction of *GOB* activity by downregulating several *SPL/SBPs* and mutated *PRO* in the same plant may lead to initiation of multiple partially fused aberrant carpels that originate the fruit-like organs in these genotypes.

Two major *loci* involved in tomato domestication are related to increased fruit size and number of locules: *FASCIATED* (*FAS*) and *LOCULE NUMBER* (*LC*). Both *lc* and *fas* mutants produce fruits with increased number of locules, and they synergistically contribute to fruit phenotypes extremely high locule number (Muños et al., 2011; Rodríguez et al., 2011). These genes were later cloned, and *fas* was shown to bear a mutation negatively affecting *CLAVATA3* expression, while *lc* was due to two Single Nucleotide Polymorphisms (SNPs) downstream *WUSCHEL* (*WUS*) that induced *WUS* expression (Munos et al., 2011; Xu et al., 2015a). Both mutations lead to increased meristem size. The changes in meristem size reported in *pro*, 156OE, rSBP3 and rSBP15 are probably associated with the number of locules produced by fruits, as genotypes with larger meristems produced more locules in the fruits and genotypes with smaller meristem produced simpler fruits, with reduce locule number. The downstream targets of *SPL3* and *15* that may control meristem size remains unknown. Interestingly, it was recently shown that miR156-targeted *SPL* transcription factors control shoot apical meristem (SAM) size by promoting *WUS* expression independent of *CLV1-CLV3* signalling (Fouracre & Poethig, 2019). Loss of miR156/157 function lead to the production of increased SAM, and loss of *SPL2/9/10/13* and *SPL15* in sextuple mutants induced increases in SAM size. We are currently analysing whether this mechanism is conserved in the establishment of tomato floral meristem size.

The relationship between miR156 targets and GA signalling is complex and so far, most available information was unveiled in *Arabidopsis*, mainly concerning the control of flowering time. Data from our previous study is consistent with the hypothesis that miR156 pathway modulates GA responses in the control of flowering time, although it was not demonstrated functionally (Silva et al., 2019). Here we provide functional evidence that miR156-targeted *SBP15* blocks GA effects in tomato plants, with evidences on plant architecture and fruit morphology. It was recently shown that the upregulation of rice Os*SPL14* (*SBP15* ortholog) also blocks GA effects in seed germination, seedling growth and disease susceptibility (Liu et al., 2019).

DELLA proteins were already reported to control inflorescence meristem size in *Arabidopsis* by targeting the cell cycle regulator *KRP2,* without interfering with the *WUSCHEL-CLAVATA* pathway (Serrano-Mislata et al., 2017). Like in tomato, DELLA loss of function plants exhibited increased meristem size. The downstream targets of *PRO* and *SBP15* remain uncharacterized in tomato meristem maintenance. If *SBP15* controls *WUS* (as in rice) and *PRO*

controls meristem size without affecting *CLV-WUS*, it is reasonable to predict that miR156-targeted *SBP15* and *PRO* synergistically control meristem size. Furthermore, *SBP15* controls the response to GA. Thus, the overexpression of miR156 in a background with loss of *PRO* function or increased GA levels may lead to even more increased meristem size due to increased GA responses, adding another layer of complexity in the relationship between miR156 and GA pathways. Synergic effect may also occur in double mutants when the effect of one of the mutations confer more sensitivity to the effects of the other (Pérez-Pérez, Candela, & Micol, 2009).

Cell size had a significant contribution in meristem size variations seen in 156OE, *pro*, rSBP3 and rSBP15 plants Molecular regulation of cell size homeostasis in plant meristems remains largely unknown. It was proposed that cell size in *Arabidopsis* SAM is regulated by a balance between cell growth and cell division. Also, the homeostatic size of the cells in meristematic tissues depends on cell growth rate (Jones et al., 2017). MiR156 module controls plastochron length, and double mutants *spl9 spl15* were shown to have shorter plastochrons (Wang et al., 2008). The reasons why miR156 and GA control cell size in the floral meristem remain an opened question. However, studying the relationship between organ initiation rate, cell growth and cell division may provide interesting insights.

This is the first study to date that explores the complex relationship between miR156 pathway and GA in the control of gynoecium development. It will be interesting to understand how *SBP15* controls GA responses at cell and molecular levels. It will shed light on much of the fruit phenotype reported in 156OE is due to increased sensitivity to GA and how much is related to the downregulation of other *SBP*s.

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2.5. Supplemental material

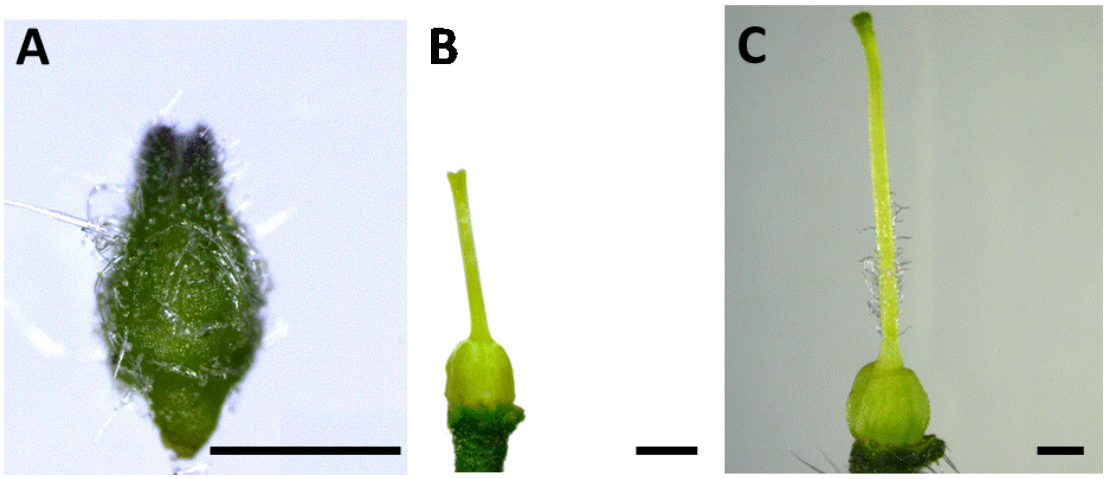

Figure S1. Developmental stages collected for RT-qPCR analysis. (A) Early flower buds at stages 6 to 8, according to Brukhin et al. (2003), here referred as CB. Bar = 1mm. (B) Developing gynoecium from dissected stage 10 floral bud. Bar = 2mm. (C) Fully developed ovary dissected from an yellow opened flower at anthesis. This final developmental stage was not collected for RT-qPCR analyses. Bar = 2mm

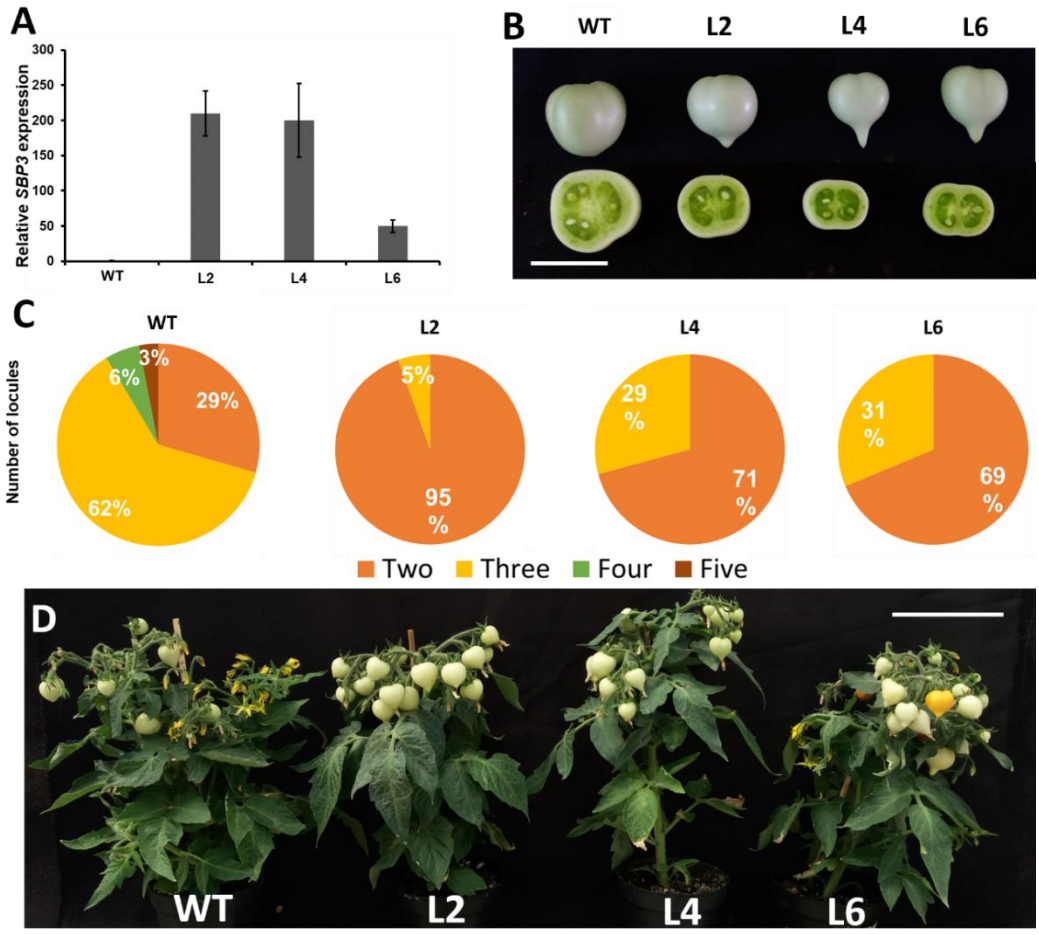

Figure S2. Molecular and morphological characterization of rSBP3 overexpression lines. (A) Relative SBP3 transcript levels in leaves of each line. Tomato TUBULIN (Solyc04g081490) was used as an internal control for all experiments. qRT-PCR values are mean ± s.e. of three biological replicates. (B) Representative fruits of WT, and rSBP3 lines L#2, L#4 and L#6. Bar= 2cm (C) Percentage of fruits producing different number of locules in each genotype (n = 20 plants). (D) Representative plants of each line. Bar= 7cm

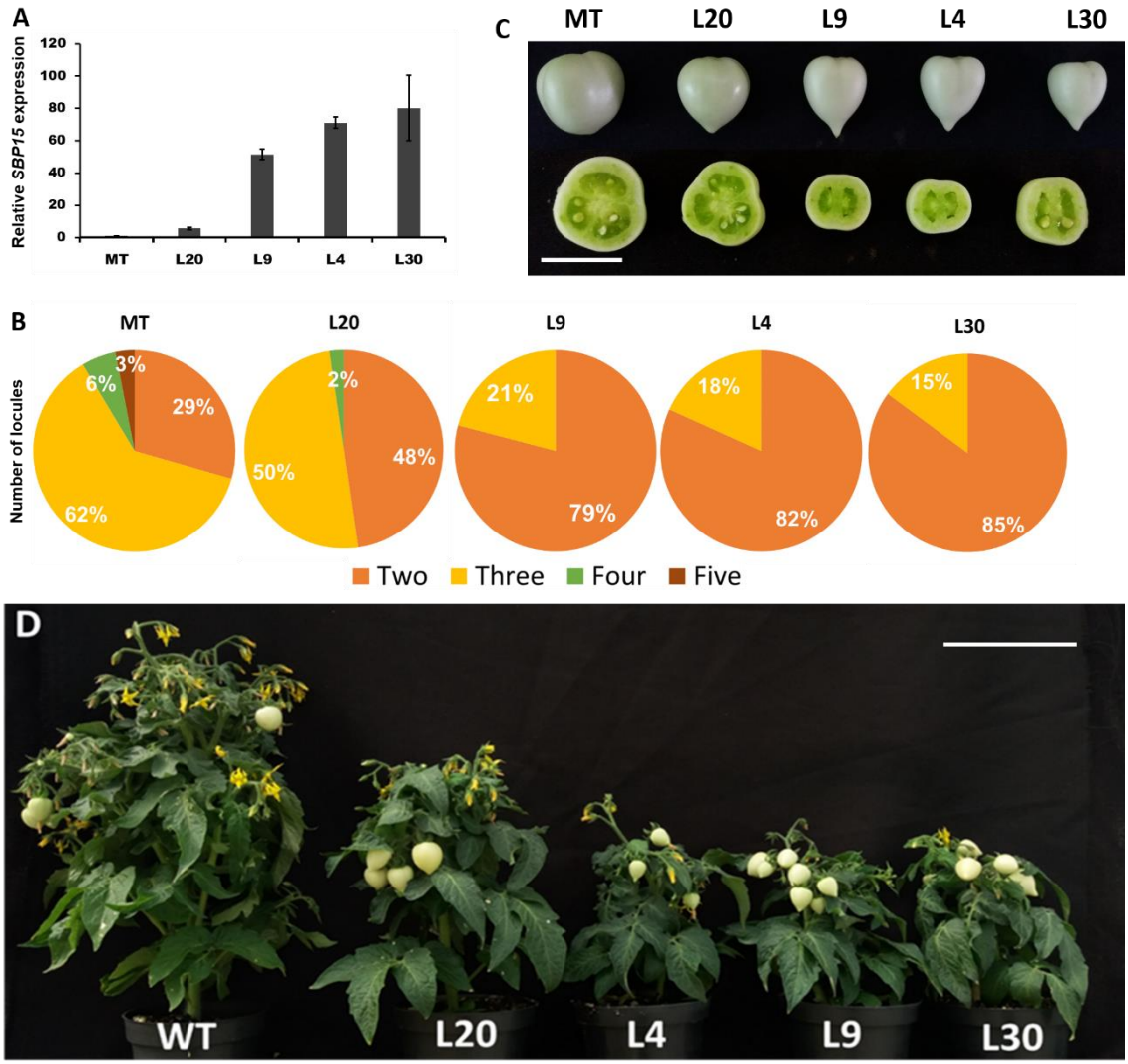

Figure S3. Molecular and morphological characterization of rSBP15 overexpression lines. (A) Relative SBP15 transcript levels in leaves of each line. Tomato TUBULIN (Solyc04g081490) was used as an internal control for all experiments. qRT-PCR values are mean ± s.e. of three biological replicates. (B) Representative fruits of WT, and rSBP15 lines L#20, L#4, L#9 and L#30. Bar= 2cm(C) Percentage of fruits producing different number of locules in each genotype. (n = 150 fruits) (D) Representative plants of each line. Bar= 7cm.

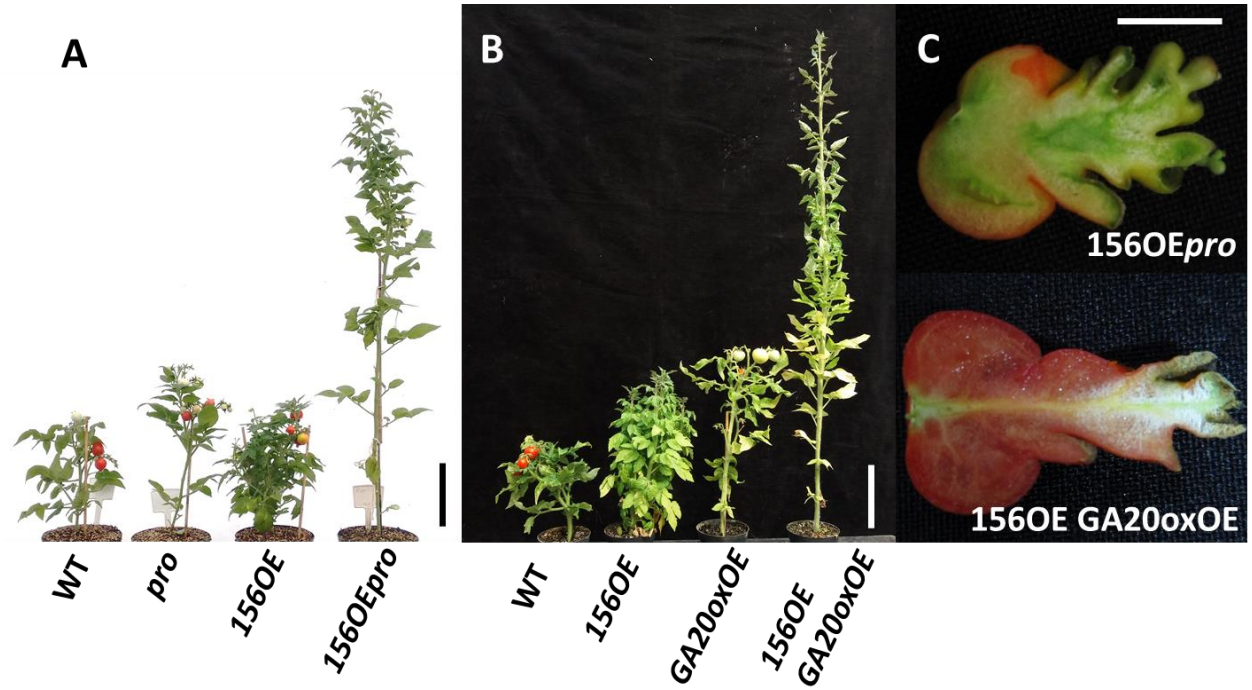

Figure S4. Overexpression of miR156 in a background with mutated PRO or increased GA levels significantly alters plant architecture and fruit morphology. (A) Representative WT, pro, 156OE, and 156OE;pro plants. (B) Representative WT, 156OE, GA20oxOE and 156OE;GA20oxOE. Bars = 7cm. (C) Longitudinal section of 156OE;pro and 156OE; GA20oxOE fruits showing no obvious locule structure. Bar = 1.5cm.

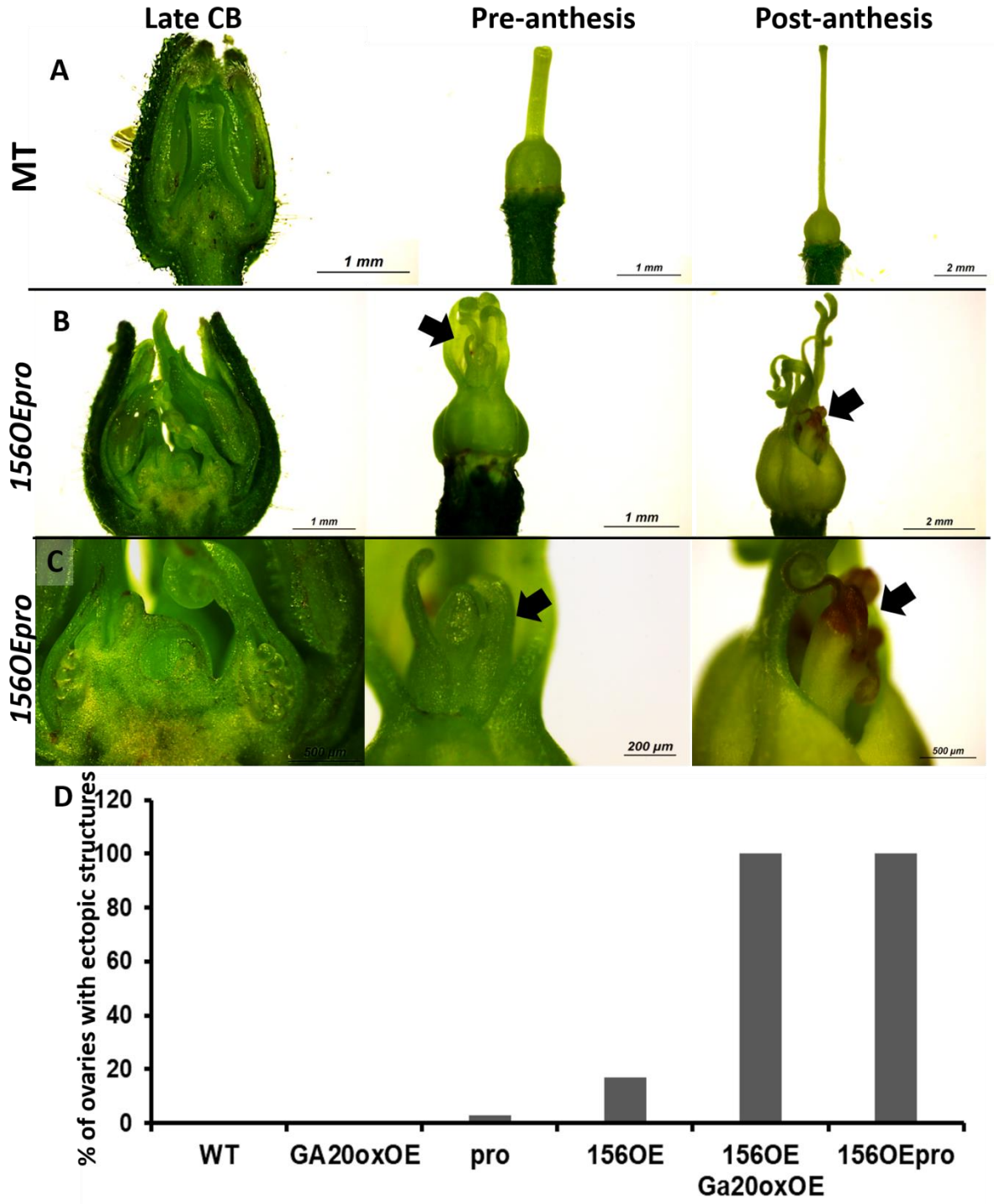

Figure S5. Stereomicroscope images of closed buds at later stages (in transverse sections), pre-anthesis ovaries and post-anthesis ovaries of MT (A) and 156OE;pro (B and C). (D) Percentage of the ovaries from the first inflorescence that showed indeterminate ectopic in each genotype. Black arrows indicate indeterminate ectopic structures growing from the ovary.

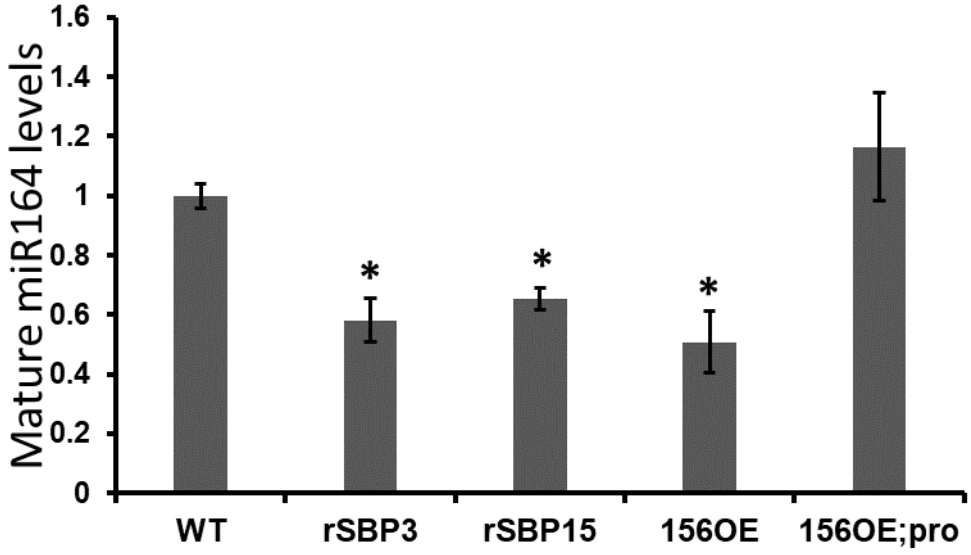

Figure S6 Mature miR164 levels in closed flower buds (CB) of WT, rSBP3, rSBP15, 156OE and 156OE;pro. Tomato TUBULIN (Solyc04g081490) was used as an internal control for all experiments. qRT-PCR values are mean ± s.e. of three biological replicates. Asterisks indicate a significant difference when compared with reference sample according to Student's t test (two tailed; *$p<0.05$).

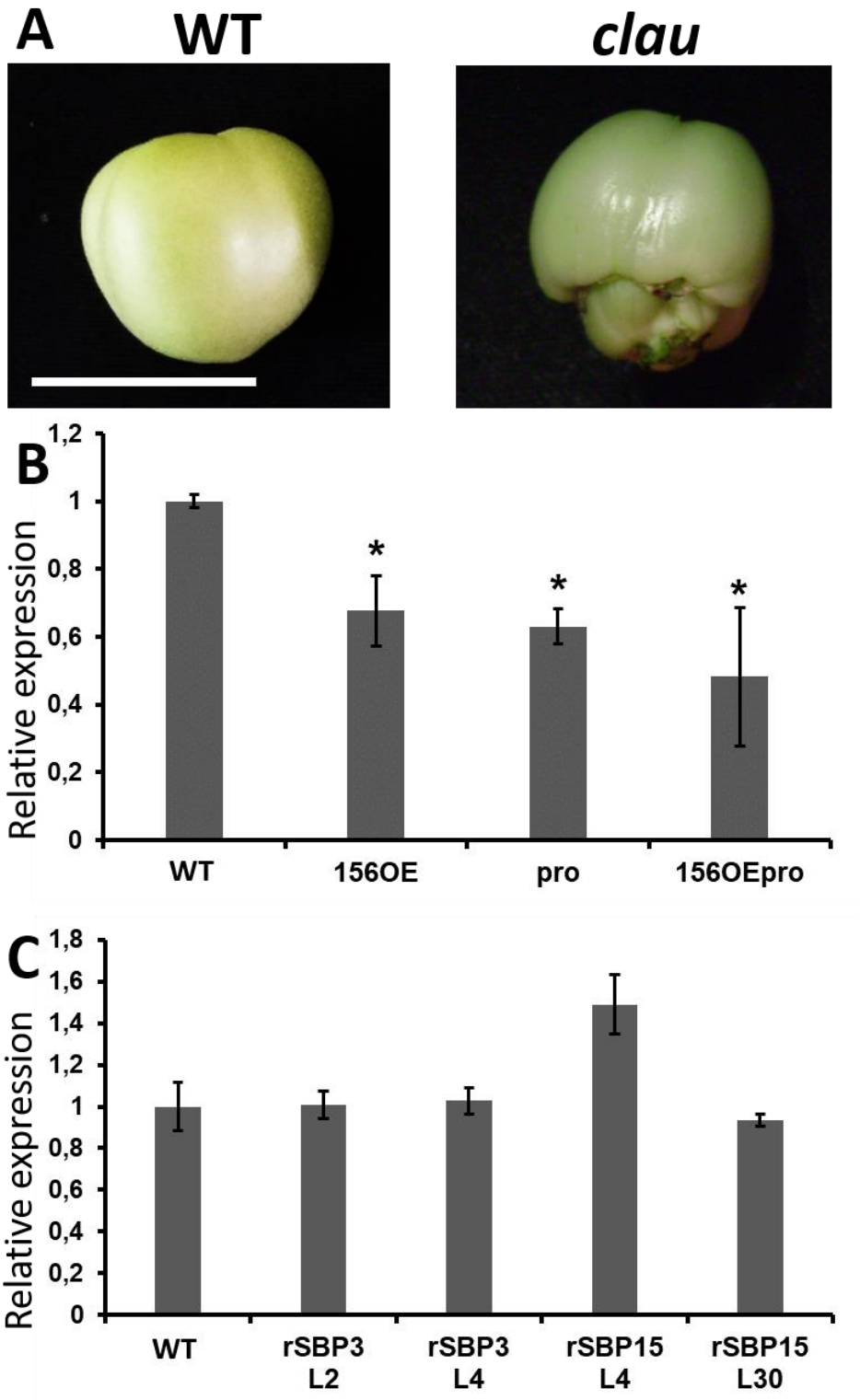

Figure S7. CLAUSA may be involved in 156OE phenotype, but not via SBP3 or SBP15. (A) clau mutant fruit phenotype. Bar= 2cm (B) and (C) Relative CLAU expression in closed flower buds (CBs) of WT, 156OE, pro, 156OE;pro and two independent lines of and rSBP15. Tomato TUBULIN (Solyc04g081490) was used as an internal control for all experiments. qRT-PCR values are mean ± s.e. of three biological replicates. Asterisks indicate a significant difference when compared with reference sample according to Student's t test (two tailed; *$p<0.05$).

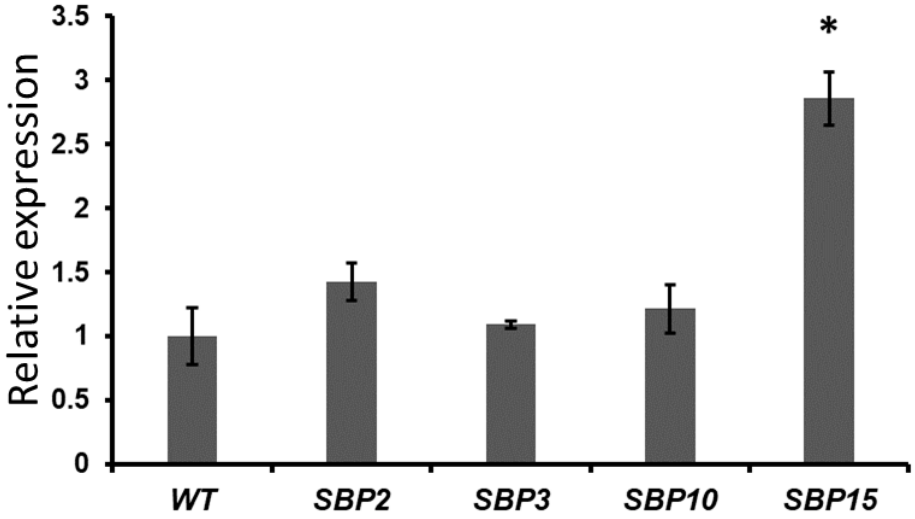

Figure S8. Expression of some miR156-targeted SBPs in procera closed flower buds. Tomato TUBULIN (Solyc04g081490) was used as an internal control for all experiments. qRT-PCR values are mean ± s.e. of three biological replicates. Asterisks indicate a significant difference when compared with reference sample according to Student's t test (two tailed; *p<0.05).

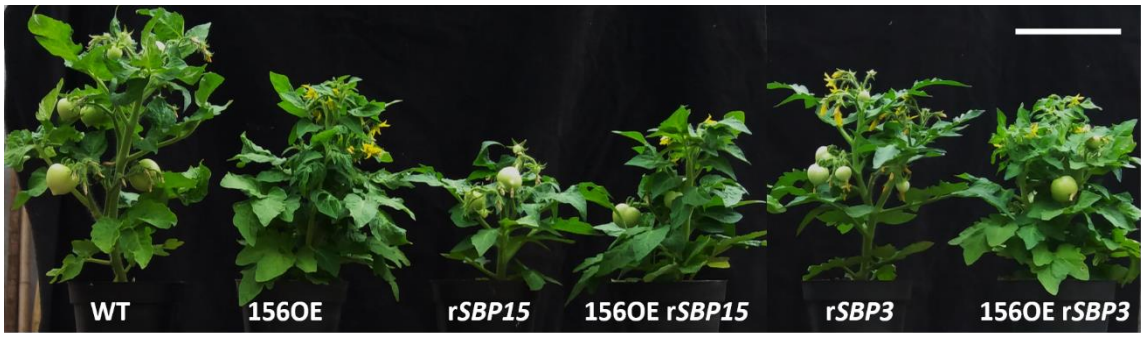

Figure S9. Representative WT, 156OE, rSBP15, 156OE;rSBP15, rSBP3 and 156OE;rSBP3 plants used in fruit and ovary morphological analysis. Bar = 7cm.

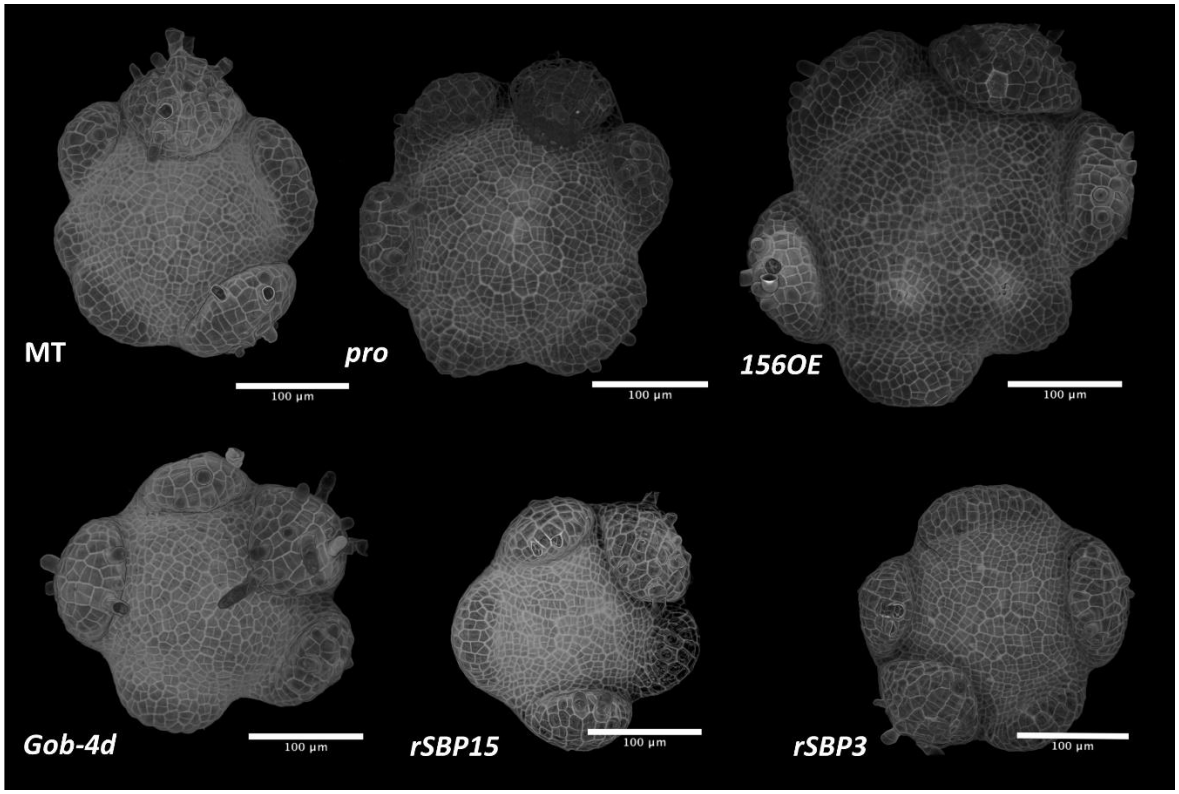

Figure S10. Raw 3D reconstruction of apexes stained by mPS-PI. The same representative meristems shown in Figure 7.

Table 1. Oligonucleotide sequences used in this work

Primer	Sequence	Locus ID	Utilization
rSBP3F	CACCATGGACTCCGACAGCGTTTCG	Solyc10g009080	Cloning
rSBP3 R	TTACATGTGAAACACAGGATAG	Solyc10g009080	Cloning
RSBP15 F1 F	CACCATGGAACTGGGTTCAGTGTCT	Solyc10g078700	Cloning
rSBP15 F1 R	GCTTAACAAGCTTAGAGCACCACTT GAATCTGAGACTCCAG	Solyc10g078700	Cloning
rSBP15 F2 F	GTGCTCTAAGCTTGTTAAGCAAT CGGTCCTGGGGATCAAGG	Solyc10g078700	Cloning
rSBP15 F2 R	TCAAAGAGTCCAGTGCACATTCTG	Solyc10g078700	Cloning
GOB clon F	CACCGAGATTTATCATCAGATGCAGTTTG	Solyc07g062840	Cloning
GOB clon R	TCAGTAGCTCCACATACAGTCAAGT	Solyc07g062840	Cloning
PRO clon F	CACCAAGAGAGATCGAGATCGAGATCG	Solyc11g011260	Cloning
PRO clon	TTACAACTCGACTTCTCCGGC	Solyc11g011260	Cloning
GOB F	GGTTCTGGACTGCAACTTCAC	Solyc07g062840	RT-qPCR
GOB R	CCATTTTCGCTTTCCCAGG	Solyc07g062840	RT-qPCR
SBP3 F	CAAGTTGAACGGGCACCTAC	Solyc10g009080	RT-qPCR
SBP3 R	TGGCAAATGACAGAAGAGAGAG	Solyc10g009080	RT-qPCR
SBP15 F	GGTTCAGCTACCAGGACCAG	Solyc10g078700	RT-qPCR
SBP 15 R	TGTGAACTTGGCTGTTGACC	Solyc10g078700	RT-qPCR
SBP2 F	CCCCTTGTCCACTCTAAACCTAC	Solyc04g045560	RT-qPCR
SBP2 R	GTCTGCATCTCAGTGGTCCCT	Solyc04g045560	RT-qPCR
SBP10 F	TTTTAATCGAGGTGCCAAGG	Solyc05g015510	RT-qPCR
SBP10 R	AACACTCTCAGGCTCGGATG	Solyc05g015510	RT-qPCR
miR164 F	ACTGATGTGGAGAAGCAGGGCA	MIMAT0000185	RT-qPCR
miR156 F	CCTGAGTGACAGAAGAGAGTG	MIMAT0000167	RT-qPCR
Reverse Universal	GTGCAGGGTCCGAGG	----------	RT-qPCR
SlTUB F	AACCTCCATTCAGGAGATGTTT	Solyc04g081490	RT-qPCR
SlTUB R	TCTGCTGTAGCATCCTGGTATT	Solyc04g081490	RT-qPCR
miR164 RT	GTCGTATCCAGTGCAGGGTCCGAG GTATTCGCACTGGATACGACTGCACG	MIMAT0000185	Reverse Transcription
miR156 RT	GTCGTATCCAGTGCAGGGTCCGA GGTATTCGCACTGGATACGACTGCTCT	MIMAT0000167	Reverse Transcription

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