University of São Paulo
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The role of microRNA156-regulated *SQUAMOSA PROMOTER BINDING PROTEINS* in the control of shoot branching in tomato

Diêgo Armando Pinheiro Brito

Dissertation presented to obtain the degree of Master in Science. Area: Plant Physiology and Biochemistry

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Bachelor of Biotechnology

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Advisor:
Prof. Dr. **FABIO TEBALDI SILVEIRA NOGUEIRA**

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CONTENTS

RESUMO

O papel das SQUAMOSA PROMOTER BINDING PROTEINS reguladas pelo microRNA156 no controle da ramificação lateral em tomateiro

A formação de ramos laterais é um dos principais aspectos da arquitetura vegetativa, determinando a forma de crescimento da planta e tendo efeitos diretos sobre a produtividade. Ramos laterais são originados a partir de meristemas axilares, formados nas axilas de cada primórdio foliar. Uma vez formados, meristemas axilares dão origem a gemas axilares, compostas por pequenas folhas não expandidas. Dependendo do programa de desenvolvimento da planta e de condições ambientais as gemas podem permanecer dormentes ou brotarem, dando origem a um novo ramo. Diversos fatores de transcrição, fitohormônios, metabólitos e sinais ambientais já foram descritos como sendo importantes para a formação de meristemas axilares ou de regulação da atividade das gemas axilares. Dentre estes, os fatores de transcrição da família *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPLs*/*SBPs*) regulados pelo microRNA156 (miR156) emergiram como potenciais reguladores da arquitetura vegetativa, regulando desde a formação do meristema axilar quanto à atividade das gemas. Dessa forma, neste trabalho, nós: (1) revisamos a literatura disponível sobre os principais sinais endógenos e ambientais crontrolando a fomação de meristemas axilares e atividades das gemas axilares e; (2) estudamos o papel das *SlSBPs* reguladas pelo miR156 no controle da ramificação lateral em tomateiro, demonstrando uma interação entre o modulo miR156/SBP e a sinalização dos fitohormônios auxina e citocinina, além de uma possível interação com outros fatores de transcrição envolvidos na regulação da atividade de gemas axilares.

Palavras-chave: Tomateiro, Ramificação lateral, Meristemas axilares, Gemas axilares, MicroRNA156, SPLs/SBPs, Auxina, Citocinina

ABSTRACT

The role of microRNA156-regulated *SQUAMOSA PROMOTER BINDING PROTEINS* in the control of shoot branching in tomato

The formation of branches is one of the key determinants of shoot architecture, determining how plants grow and having direct impacts in their productivity. Lateral branches originate from axillary meristems, formed in the axils of each leaf primordia. Once established, axillary meristems give rise to a few lead primordia, giving rise to an axillary bud. Depending on the plants developmental program and on environmental conditions, axillary buds may remain dormant or outgrow, forming a new lateral branch. Several transcription factors, phytohormones, metabolites and environmental signals have been associated with the formation of axillary meristems or with the regulation of axillary bud activity. Members of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPLs*/*SBPs*) family of transcription factors regulated by the microRNA156 (miR156) have emerged as potential regulators of shoot architecture, regulating both the formation of axillary meristems and the activity of axillary buds. In this work, we: (1) review recent literature on the effects of the main endogenous and environmental signals controlling the formation of axillary meristems and activity of axillary buds and; (2) investigated the role of miR156-targeted *SlSBPs* in the control of shoot branching in tomato, demonstrating an interaction between the miR156/SBP module and auxin and cytokinin signalling, besides a possible interaction with other transcription factors controlling the activity of axillary buds.

Keywords: Tomato, Shoot branching, Axillary meristems, Axillary buds, MicroRNA156, SPLs/SBPs, Auxin, Cytokinin

1. GENETIC AND PHYSIOLOGICAL REGULATION OF SHOOT BRANCHING

ABSTRACT

Unlike animals, plants are highly plastic organisms, being able modulate their shoot and root architecture depending on endogenous or environmental signals. Much of this plasticity is due to the activity of meristems, groups of stem cells which may differentiate into any plant organ. The formation of branches, for instance, depends on the activity of secondary meristems located in the axils of each leaf, known as axillary meristems (AMs). AMs initiate axillary buds (ABs) which may or not elongate into a lateral branch. Here we review the role of transcription factors, phytohormones, metabolites and environmental signals controlling AM formation and AB activity, with a special focus on the role of the microRNA156-regulated SQUAMOSA PROMOTER-BINDING PROTEINs (SPLs) which seems to integrate both steps of the formation of branches.

1.1. Introduction

During plant development, the formation of branches is one of the key steps in the establishment of the shoot architecture. After embryogenesis, the shoot apical meristem (SAM) gives rise to all aerial organs of a plant, through the production of a series of phytomers, consisting of an internode, a leaf and a secondary SAM at the axil of each leaf, the axillary meristem, which have the same developmental potential as the primary SAM. Axillary meristems usually initiate a few unexpanded leaves, forming structures known as axillary buds. These ABs may then remain dormant or continue to expand to form a branch, establishing a new axis of growth (Domagalska and Leyser, 2011). Thus, the final shoot architecture depends on the formation of axillary meristems and the activity of the axillary buds. These two steps are strictly controlled by endogenous signals such as transcription factors, phytohormones and metabolites, but also by exogenous factors such as light and nutrient availability.

1.2. Axillary meristem formation

There are two long-standing models for the cellular origin of axillary meristems. The "*de novo* induction" model proposes that axillary cells originate from shoot cells (either internode or leaf) which dedifferentiate to form the meristematic cells of the AM (Steeves and Sussex, 1989; McConnell and Barton, 1998). On the other hand, according to the "detached meristem" model, the AM progenitor cells are set apart early in the primary shoot apical meristem and retain their

meristematic state until the formation of an axillary meristem. Recent studies reinforced the "detached meristem" hypothesis, by tracing through live-imaging microscopy a meristematic cell lineage in the boundary between the leaf primordia and the SAM which later will form the AMs (Burian et al., 2016; Shi et al., 2016). Although this cell lineage is specified early, AMs only become morphological defined structures at a distance from the SAM, in the axils of primordia 21 (P21) in *Arabidopsis thaliana* (Greb et al., 2003) and P7 in tomato cv. Moneymaker (Wang et al., 2014a).

1.2.1. Transcription factors associated with AM formation

The boundaries between different plant organs are zones of reduced cell division and expansion and their formation is regulated by several transcription factors and phytohormones, many of which also regulate meristem initiation (Wang et al., 2016). The members of the NAC (NAM, ATAF1/2, and CUC2) protein family *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2* and *CUC3* in Arabidopsis and their ortholog *GOBLET* (*GOB*) in tomato have been extensively described for their role in the formation of boundaries. (Aida et al., 1997; Vroemen et al., 2003; Berger et al., 2009). The loss-of-function of these genes causes abnormalities such as impaired SAM development, the fusion of cotyledons and floral organs and simplified leaves (Aida et al., 1997; Vroemen et al., 2003; Brand et al., 2007). *CUC1/2/3* and *GOB* transcripts accumulate in leaf axils (Vroemen et al., 2003; Raman et al., 2008; Rossmann et al., 2015) and it has been reported that mutants in these genes are also affected in AM formation (Hibara et al., 2006; Raman et al., 2008; Busch et al., 2011). While *cuc3* mutants frequently lack AMs, knocking down *CUC1* and *CUC2* in the *cuc3* background completely abolished AM formation, resembling the *gob-3* mutant which completely lack AMs (Raman et al., 2008; Busch et al., 2011). CUC1 and CUC2 are post-transcriptionally regulated by the microRNA164 (Laufs et al., 2004) and the absence of this regulation, either in knock-out mutants for the miR164 precursor genes or in plants harbouring miR164-resistant *CUC1/2*, leads to the formation of secondary axillary meristems (Raman et al., 2008).

It has been proposed that *CUC1/2* and *GOB* control AM development, at least in part, by regulating the expression of the GRAS transcription factor *LATERAL SUPPRESSOR* (*LS* in tomato, *LAS* in Arabidopsis*;* Schumacher et al., 1999; Greb et al., 2003). *LS/LAS* expression domain overlaps with that of *CUC1-3/GOB* in leaf boundaries, and their loss-of-function mutants are unable to form axillary meristems during vegetative development (Malayer and Guard, 1964; Greb et al., 2003; Busch et al., 2011; Rossmann et al., 2015). After floral transition, however, the *las* mutant initiate AMs in the axils of cauline leaves, whereas in the tomato *ls* mutant, AMs are

formed in the axils of the first two leaves below the inflorescence (Malayer and Guard, 1964; Greb et al., 2003). Recently, a genome-wide protein-DNA interaction study has identified the *LAS* promoter as a regulatory hub where several transcription factors bind to regulate its expression, including type B ARABIDOPSIS RESPONSE REGULATORS (ARRs) as ARR1, NAC proteins such as CUC2 and several members of the SQUAMOSA PROMOTER BINDING PROTEINS (SPLs), including SPL9 and SPL15 (Tian et al., 2014). While SPL9 and SPL15 repress, ARR1 and CUC2 activate *LAS* transcription, corroborating previous reports (Hibara et al., 2006; Raman et al., 2008; Tian et al., 2014).

The CUC2 promoter is also targeted by several transcription factors, including the R2R3 MYB transcription factor REGULATOR OF AXILLARY MERISTEMS 1 (RAX1) and RAX3 (Tian et al., 2014), corroborating the previous observation that *CUC2* expression is diminished in *rax1* mutants (Keller et al., 2006). In Arabidopsis, *RAX* genes act redundantly to control the establishment of axillary meristems, with *RAX1* being more required in the early stages of vegetative development and *RAX2* and *RAX3* more important for AM formation from middle to late vegetative development (Müller et al., 2006). In tomato, the orthologous of *RAX1*, *BLIND* (*BL*), control axillary meristem formation during both vegetative and reproductive development (Schmitz et al., 2002). Unlike *ls* mutants, the formation of AMs in *blind* mutants is not completely inhibited, as they are able to form a reduced number of branches, which is due to the redundant function of other *BL*-like genes (Schmitz et al., 2002; Busch et al., 2011).

Transcription factors downstream of *LAS* have also been implicated in the AM formation gene regulatory network. It has been demonstrated by mutant screens that *LAS* acts upstream of the HD-ZIP III transcription factor REVOLUTA (REV) (Greb et al., 2003). *REV* is broadly expressed in shoot and root organs, including leaf axils, and *rev* mutants usually fail to produce AMs during both vegetative and reproductive development (Talbert et al., 1995; Otsuga et al., 2001). In *las* mutants, *REV* expression pattern in the SAM and vascular bundles is unaltered, but its expression in axillary meristems is drastically reduced, indicating that LAS activates *REV* expression in AMs (Greb et al., 2003). *REV* is post-transcriptionally regulated by the microRNA165/166 and mutations in the miR165/166 recognition sites cause the ectopic production of branches in Arabidopsis and ectopic flowers and fruits in tomato (Zhong and Ye, 2004; Hu et al., 2014).

Recently, it has been found that *REV* directly activates the expression of *SHOOT MERISTEMLESS* (*STM*) (Shi et al., 2016), a transcription factor required for initiation and maintenance of meristematic cells. *STM* is expressed in the embryo since the globular stage, in the cells which will form the SAM (Long et al., 1996). Likewise, *STM* expression can also be detected

very early in the leaf axil meristematic cells and is maintained until AM formation (Long and Barton, 2000; Grbić and Bleecker, 2000; Greb et al., 2003). The weak *STM* mutant *stm-bum-1* shows reduced initiation of AMs and laser ablation of *STM*-expressing cells abolished axillary meristem initiation, indicating that STM is required to maintain a stem cell population in the leaf axil (Shi et al., 2016). In tomato, ectopic expression of *Tkn2*, the ortholog of *STM*, leads to the formation of ectopic shoots and accessory axillary buds (Parnis et al., 1997; Janssen et al., 1998; Busch et al., 2011).

WUSCHEL (*WUS*) is a homeodomain transcription factor, which, together with *CLAVATA3* (*CLV3*), is required for the maintenance of the stem cell niche in the SAM (Mayer et al., 1998; Fletcher et al., 1999). *WUS* and *CLV3* are expressed very early in embryonic development in the cells which will become the SAM, and also during AM initiation (Xin et al., 2017). Recent evidence shows that *WUS* is required for the initiation of axillary meristems, as *wus* mutants, besides showing severe SAM defects, are also impaired in AM formation in both rice and Arabidopsis (Tanaka et al., 2015; Wang et al., 2017; Xin et al., 2017). After the establishment of the WUS-CLV feedback loop, the AMs have the same developmental potential as the SAM.

1.2.2. Phytohormones involved in AM formation

In addition to transcription factors, phytohormones also play an important role during AM formation. The establishment of boundary zones in different parts of the plant body is commonly associated with low auxin concentrations (Wang et al., 2016). It has been observed in both Arabidopsis and tomato that emerging leaf primordia show an auxin maxima while leaf axil cells are usually depleted from auxin (Wang et al., 2014a, 2014b). Reduced auxin transport, either in loss-of-function mutants in auxin exporter proteins or by treating plants with the auxin transport inhibitor 1-Naphthylphthalamic acid (NPA), impairs the formation of axillary meristems (Wang et al., 2014a, 2014b). Furthermore, ectopic expression of an auxin biosynthesis gene under the control of *LAS* or *CUC2* promoters was able to suppress AM initiation, suggesting that an auxin minimum is indeed required for AM initiation (Wang et al., 2014b, 2014a). Ectopic auxin synthesis in leaf axils, significantly reduce the expression of *STM*, indicating that an auxin minimum is required for the maintenance of the stem cell population in leaf axils (Wang et al., 2014a, 2014b; Shi et al., 2016).

Mutants defective in cytokinin biosynthesis and perception are also affected in AM formation, suggesting cytokinin is required for proper establishment of AMs (Wang et al., 2014b; Müller et al., 2015). It has been observed that following the establishment of an auxin minimum in leaf axils, a cytokinin pulse occurs before AM initiation (Wang et al., 2014b). This cytokinin pulse is required for the *de novo* activation of *WUS* expression in AMs, as ARR1 directly binds to the

WUS promoter to activate its transcription (Wang et al., 2017). In the SAM, *STM* promotes the expression of cytokinin biosynthesis genes (Jasinski et al., 2005; Yanai et al., 2005). Similarly, the cytokinin pulse preceding the AM formation may also be a result of *STM* function.

Most recently, it has been proposed that gibberellins (GA) also play a role in the formation of axillary meristems as either exogenous GA treatment or ectopic GA biosynthesis in leaf axils reduce the ration of AM formation (Zhang et al., 2020b). DELLA proteins are negative regulators of GA response, and quintuple *della* loss-of-function mutants also show impaired AM formation (Zhang et al., 2020b). It has been shown that DELLA act by directly interacting with *SPL9* and blocking its function as a negative regulator of *LAS* expression (Tian et al., 2014; Zhang et al., 2020b).

1.3. Axillary bud outgrowth

Axillary meristems have the same developmental potential as the primary shoot apical meristem. They usually give rise to a few unexpanded leaves, forming an axillary bud. This axillary bud may then remain dormant or give rise to new phytomers to form a branch. This process is tightly regulated by several endogenous or exogenous signals, such as phytohormones, transcription factors, light and nutrient availability which may act either locally in the buds or at a distance to control their activity.

1.3.1. Auxin

The common observation that removing the shoot tip of a plant induces the outgrowth of lateral branches led to the formulation of the apical dominance theory. According to this theory, the inhibitory effect of the shoot tip was either due to an inhibitory substance synthesized in the apex and transported down the shoot or by the competition for nutrients between the growing apex and the axillary buds (Snow, 1925). The nature of this inhibitory substance was discovered by Thimann and Skoog (1933), who found that applying auxin to decapitated plants was able to restore the inhibition exerted by the shoot apex. Conversely, applying an auxin transport inhibitor on the stem caused the same effects of decapitation (Snyder, 1949).

Auxins are mostly synthesized in actively growing leaf primordia of the shoot apex and is transported down the stem partly through a process known as Polar Auxin Transport Stream (PATS, Domagalska and Leyser, 2011). The PATS is mediated by at least three group of membrane

proteins: AUX1, which act as an auxin influx carrier (Bennett et al., 1996) as well as ABCBs (Noh et al., 2001) and PIN-FORMED proteins (Gälweiler et al., 1998), which act as auxin efflux carriers. Auxin is able to regulate its own efflux through PIN proteins, either by up-regulating their expression or inhibiting their endocytosis (Vieten et al., 2005; Paciorek et al., 2005). In the stem, the PATS is mostly driven by PIN1, which usually locate in the basal plasma membrane of xylem parenchyma cells, driving the auxin flux rootward (Gälweiler et al., 1998; Bennett et al., 2016).

The observation that apically-derived auxin does not enter the buds (Booker et al., 2003) led to the formulation of two non-exclusive models to explain how auxin inhibit bud activity. The auxin transport canalization model is based on the assumption that axillary buds need to export their auxin to outgrow and this is limited by the strength of the bud as an auxin source and the sink strength of the PATS in the main stem (Li and Bangerth, 1999; Balla et al., 2011). Therefore, decapitating the shoot apex enhances the sink strength of the stem and allows the axillary buds to establish its auxin export (Domagalska and Leyser, 2011).

The second model of bud inhibition proposes that auxin acts through a second messenger, which it is assumed to be either cytokinins or strigolactones, phytohormones shown to directly activate and repress bud outgrowth, respectively (Domagalska and Leyser, 2011). Decapitation induces while exogenous auxin represses cytokinin biosynthesis genes in both pea and Arabidopsis (Tanaka et al., 2006; Müller et al., 2015). Exogenous auxin induces the expression of *MORE AXILLARY GROWTH3* (*MAX3*) and *MAX4*, two enzymes that belong to the biosynthetic route of strigolactones (Hayward et al., 2009).

1.3.2. Strigolactones

Strigolactones (SL) are carotenoid-derived molecules, long known for their function in promoting the colonization of parasitic weeds and arbuscular mycorrhiza fungi in the plant roots (Cook et al., 1966; Akiyama et al., 2005). Their role as a phytohormone was only recently proposed (Gomez-Roldan et al., 2008; Umehara et al., 2008), and since then, several discoveries regarding its role in plant development have been made, including functions in root development, leaf shape, stem elongation and shoot branching (Rameau et al., 2019).

Strigolactones are synthesized from carotenoid precursors by a series of enzymes including the DWARF27 (D27) isomerase, the carotenoid cleavage dioxygenase 7 (CCD7, encoded by MAX3 in Arabidopsis and D17 in rice) and CCD8 (encoded by MAX4 in Arabidopsis and D10 in rice), and the cytochrome P450 MAX1 (Domagalska and Leyser, 2011). Strigolactones are then perceived by the putative receptor D14, which physically interacts with MAX2/D3, a component

of the SCF ubiquitin-ligase complex (Rameau et al., 2019). The SL-D14-MAX2 complex promotes the polyubiquitination and the consequent degradation by the proteasome of the SL signalling repressors, D53 in rice (Jiang et al., 2013; Zhou et al., 2013) and its orthologs SUPRESSOR OF MAX2 1-LIKE6 (SMXL6), SMXL7 and SMXL8 in Arabidopsis (Wang et al., 2015; Soundappan et al., 2015).

D53 and SMXL6/7/8 carry ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motifs, allowing their interaction with TOPLESS (TPL) and TPL-related (TPRs) transcriptional co-repressors (Jiang et al., 2013; Zhou et al., 2013; Wang et al., 2015; Soundappan et al., 2015). Recently, it has been shown that SMXL6/7/8 have DNA-binding activity and may act either by directly regulating their own targets, or by interacting with other transcription factors and modulating their transcriptional activity (Wang et al., 2020).

The role of strigolactones in shoot inhibition is very conserved, as mutants in SL biosynthesis and signalling across several species show a highly branched phenotype whereas treating axillary buds with synthetic SLs inhibit their outgrowth (Rameau et al., 2019). There are two non-exclusive hypotheses to explain the SL role in bud inhibition. One of them proposes that SL acts through the transcriptional activation of *BRC1* in axillary buds. In pea and rice, SL treatment cannot inhibit the highly branched phenotype of *brc1* and *tb1* mutants (Braun et al., 2012; Minakuchi et al., 2010). However, in Arabidopsis, *brc1* mutants are partially sensitive to SL inhibition (Seale et al., 2017). The second hypothesis for SL action is through the inhibition of auxin transport. Strigolactone biosynthesis mutants show enhanced PATS and PIN1 localization on cell membranes, whereas exogenous strigolactone treatment to stem segments reduce their ability to transport auxin (Crawford et al., 2010; Shinohara et al., 2013). These results suggest that SL systemically inhibits auxin transport, reducing the auxin sink strength of the stem and enhancing the competition between axillary buds (Crawford et al., 2010; Shinohara et al., 2013). Recently, it has been shown that SL inhibits the positive feedback of auxin on PIN-mediated canalization, reducing the buds ability of establishing their own auxin export (Zhang et al., 2020a).

1.3.3. Cytokinins

Cytokinins (CK) are a class of phytohormones known for inducing cell division and differerentiation and affecting several aspects of plant development (Feng et al., 2017). The role of cytokinins as promoters of bud outgrowth has been known for decades (Wickson and Thimann, 1958). CK treatment can promote axillary bud elongation in a dose-dependent manner, either when

supplied directly to the buds or to the stem (Wickson and Thimann, 1958; Dun et al., 2012; Roman et al., 2016).

Bioactive cytokinins are synthesized from adenine precursors in a biochemical pathway mainly catalysed by enzymes from two gene families: *ADENYLATE ISOPENTENYLTRANSFERASEs* (*IPT*) and *LONELY GUY* (*LOG*) (Feng et al., 2017). Plants with elevated *IPT* or *LOG* expression show an increased branching phenotype (Smigocki, 1991; Eviatar-Ribak et al., 2013) while multiple *ipt* mutants fail to initiate axillary meristems (Müller et al., 2015).

Confirming the role of cytokinins as second messengers in the auxin-mediated repression of bud outgrowth, the expression of *IPT* genes in the stem is induced upon decapitation, whereas auxin treatment inhibits their expression in pea, rice and Arabidopsis (Tanaka et al., 2006; Minakuchi et al., 2010).

In order to activate axillary buds, cytokinins act, at least partially, through the inhibition of *TB1/BRC1* genes in Arabidopsis, pea and rice (Dun et al., 2009; Minakuchi et al., 2010; Braun et al., 2012). However, in pea, *brc1* mutants show enhanced bud elongation in response to CK, suggesting CK also act through a BRC1-independent mechanism (Braun et al., 2012).

An insight on the cytokinin regulatory network in axillary bud outgrowth came from transcriptomic experiments in the woody species *Jatropha curcas*, which showed that CK induces the expression of genes encoding cell cycle regulators and involved in GA biosynthesis, whereas genes associated with GA degradation are repressed (Ni et al., 2017). Given that GA induces bud outgrowth in *J. curcas* (Ni et al., 2015), this finding suggests that CK and GA act synergistically to control shoot branching in this species.

A second mode for CK action is through the regulation of auxin transport, as CK treatment enhances the localization of PIN3, PIN4 and PIN7 proteins on the plasma membranes of xylem parenchyma cells (Waldie and Leyser, 2018), in accordance with the observation that CK treatment increases auxin export from axillary buds (Li and Bangerth, 2003).

1.3.4. Gibberellins

Gibberellins (GAs) show opposing roles in the regulation of shoot branching across different species. Mostly in woody species such as Citrus, hybrid aspen, sweet cherry, *Jatropha curcas* and papaya but also in rose and snapdragon, bioactive GAs promote bud outgrowth (Marth et al., 1956; Rinne et al., 2011; Elfving et al., 2011; Ni et al., 2015; Choubane et al., 2012). On the other hand, it has been reported that exogenous GA treatment repress bud outgrowth in pea (Scott et

al., 1967). In Arabidopsis, mutants in both GA biosynthetic and signalling genes are characterized by loss of apical dominance (Koorneef et al., 1985). In tomato, silencing GA catabolic enzymes promote the accumulation of bioactive GAs in axillary buds and the inhibition of their outgrowth (Martínez-Bello et al., 2015).

1.3.5. Sugars

Although auxin has occupied a central role in the apical dominance theory, recent evidences suggest that upon decapitation, rapid bud outgrowth occurs before any changes in auxin concentration in the surrounding stem can be perceived (Morris et al., 2005). Mason and collaborators (2014) have shown that sugars, specially sucrose, which move at a faster rate than auxin, rapidly accumulate in axillary buds after decapitation. Accordingly, exogenous sucrose treatment was able to trigger bud outgrowth in intact pea plants or excised nodes of rose, in a dose-dependent manner (Mason et al., 2014; Barbier et al., 2015). On the contrary, defoliation has been shown to inhibit bud outgrowth (Kebrom et al., 2010; Mason et al., 2014). Together, these evidences suggest that the shoot apex repress initial axillary bud outgrowth by limiting their access to photoassimilates, while auxin is required for suppressing sustained bud outgrowth (Barbier et al., 2019).

1.3.6. Environmental signals

Given that shoot branching is an energy demanding process, plants have evolved mechanisms to control bud outgrowth, depending on the local environmental conditions.

One of the environmental cues known to control branching is the soil nutrient availability. In Arabidopsis, plants grown under nitrogen deficiency show reduced branching (Jong et al., 2014), which might be explained by the interaction between nitrogen and cytokinin signalling. Nitrogen-deprived roots rapidly accumulate cytokinin after nitrate resupply (Takei et al., 2001) and nitrate has been shown to activate the expression of cytokinin biosynthetic genes, especially *IPT3*, without the need for *de novo* protein synthesis (Miyawaki et al., 2004).

Light quality also affects axillary bud outgrowth. When exposed to low red to far-red light ratio (R/FR), as when grown in proximity to neighbours, plants develop a so-called shade avoidance syndrome, which among other symptoms is characterized by enhanced stem elongation and suppression of branching (Domagalska and Leyser, 2011). Early studies have found that exposing plants for as little as five minutes of low R/FT light after a 16-hour high R/FR

photoperiod was able to strongly suppress branching in tomato, and this was correlated with a significant increase in ABA content in axillary buds (Tucker D. J., 1976). Light quality is mainly perceived by *PHYTOCHROME B* (*PHYB*), which when activated by red light, migrate to the nucleus, where it interacts and negatively regulates the activity of Phytochrome Interacting Factors (PIFs), transcription factors which regulate the response to light. *phyb* mutants in sorghum and Arabidopsis show reduced branching (Kebrom et al., 2006; Finlayson et al., 2010), whereas *pif* mutants show increase branching (Xie et al., 2017).

1.3.7. TEOSINTE BRANCHED 1

TEOSINTE BRANCHED1 (*TB1*) is a gene originally discovered in maize for its function as a repressor of shoot branching (Doebley et al., 1995, 1997; Hubbard et al., 2002). Unlike its wild ancestor teosinte, known for its highly branched phenotype, modern maize plants usually produce one or two branches (Doebley et al., 1995). This increase in apical dominance during domestication was due to the selection of a highly expressing variant of *TB1* (Doebley et al., 1997).

TB1 encodes a transcription factor of the *TEOSINTE BRANCHED1, CYCLOIDEA AND PCF TRANSCRIPTION FACTOR* (*TCP*) gene family, which is composed of genes characterized as repressors of organ growth (Cubas et al., 1999). *TB1* orthologs in other monocots and in eudicots – in which it is known as *BRANCHED1* (BRC1) - have been extensively described as negative regulators of axillary bud outgrowth (Takeda et al., 2003; Aguilar-Martinez et al., 2007; Braun et al., 2012; Martín-Trillo et al., 2011; Liu et al., 2017). *TB1/BRC1* is specifically expressed in axillary meristems and buds and in the developing vascular tissue of the bud stem (Doebley et al., 1997; Aguilar-Martinez et al., 2007; Martín-Trillo et al., 2011). The loss of function of *TB1/BRC1* cause an increase in axillary bud outgrowth (Takeda et al., 2003; Aguilar-Martinez et al., 2007; Martín-Trillo et al., 2011; Nicolas et al., 2015) whereas its ectopic expression leads to the inhibition of branching in rice (Takeda et al., 2003) or a complete growth arrest in Arabidopsis and potato (Gonzalez-Grandio et al., 2013; Nicolas et al., 2015).

BRC1 is thought to act inside axillary buds as an integrator of exogenous and endogenous signals regulating bud activity. Branch-promoting signals, such as cytokinin or sucrose downregulate *BRC1* expression (Minakuchi et al., 2010; Braun et al., 2012; Mason et al., 2014; Barbier et al., 2015; Seale et al., 2017). *BRC1* mRNA levels are also diminished upon decapitation (Aguilar-Martinez et al., 2007; Martín-Trillo et al., 2011). On the other hand, strigolactones (SLs), which are phytohormones known to inhibit bud outgrowth, can activate *BRC1* expression at least in pea and Arabidopsis (Braun et al., 2012; Dun et al., 2012; Chevalier et al., 2014). *BRC1* expression

is also induced in response to shade either by increasing planting density or by growing plants under low R:FR light, two treatments that inhibit bud outgrowth (Aguilar-Martinez et al., 2007; González-Grandío et al., 2017).

Although the gene regulatory network downstream of *BRC1* has not been fully elucidated, it has been shown that BRC1 directly bind and induce the expression of the homeodomain leucine zipper genes (HD-ZIP) *HOMEOBOX PROTEIN 21* (*HB21*), *HB40* and *HB53* (González-Grandío et al., 2017). These proteins, together with BRC1, induce the expression of *9-CIS-EPOXICAROTENOID DIOXIGENASE 3* (*NCED3*), an enzyme in the abscisic acid biosynthetic route (González-Grandío et al., 2017). ABA has been shown before to act as a negative regulator of bud activity (Yao and Finlayson, 2015), so BRC1 may act, at least in part, by inducing ABA accumulation in in axillary buds.

Despite its central role in bud inhibition, the TB1/BRC1-regulated pathway is not not the only mechanism regulating bud activity in plants. Not all axillary buds are released in Arabidopsis *brc1* mutants and in the tomato and pea mutants, only the lower buds give rise to branches (Finlayson, 2007; Martín-Trillo et al., 2011; Braun et al., 2012). Additionally, it has been observed that *brc1* and strigolactone signalling mutants have additive phenotypes, corroborating the hypothesis that strigolactones also regulate other targets to prevent branching (Braun et al., 2012; Chevalier et al., 2014; Seale et al., 2017).

1.4. The microRNA156/SPL module

Members of the *SQUAMOSA PROMOTER-BINDING PROTEIN* (*SPL* in Arabidopsis, *SBP* in tomato) gene family encode transcription factors involved in several aspects of plant development such as phase transition, plastochron, stress responses, leaf development, fruit development and shoot branching (Wang and Wang, 2015). SPL proteins contain a DNA binding domain of 76 amino acids – the SBP domain, composed of two zinc fingers and a nuclear localization signal (Klein et al., 1996). In the nucleus, SPLs/SBPs regulate gene expression by targeting binding sites composed of a GTAC core (Birkenbihl et al., 2005).

A subset of *SPLs* is post-transcriptionally regulated by the microRNA156 (miR156), establishing the miR156/SPL module (Rhoades et al., 2002). The miR156/SPL module is highly conserved in plants, having been found in mosses, ferns, gymnosperms, monocots and eudicots (Willmann and Poethig, 2007). miR156 is highly expressed during early plant development, and as the plant ages its expression declines and, consequently, *SPLs* become more abundant, defining the so-called age pathway which controls the vegetative and reproductive phase changes (Wu and

Poethig, 2006; Chuck et al., 2007). This temporal regulation of miR156 abundance is due to the regulation of *MIR156* genes at the epigenetic level. In seedlings, *MIR156A* and *MIR156C* are enriched in Histone 3 Lysine 27 acetylation (H3K27ac), a histone modification associated with active transcription, and as the plant ages the levels of H3K27ac decrease, while the repressive mark H3K27me3 increase, resulting in diminished *MIR156* transcription (Xu et al., 2015). The expression of miR156 is also controlled by environmental signals. Simulated shade induces the accumulation of PIF proteins which directly inhibit the expression of several *MIR156* genes, suggesting that the shade avoidance response which include suppressed shoot branching, may be in part mediated by miR156 and SPLs abundance (Xie et al., 2017).

Only recently the mode of action of SPLs in the regulation of transcriptional activity of downstream genes have started to be elucidated. SPL3 (and their close homologs SPL4 and SPL5) promote flowering by directly activating the expression of the flowering effectors *APETALA1* (*AP1*), *FRUITFUL* (*FUL*) and *LEAFY* (*LFY*) (Yamaguchi et al., 2009). SPL9 and its paralog SPL15 also promote flowering by directly activating the expression of *AP1*, *FUL* (Wu et al., 2009; Wang et al., 2009), while *MIR172* is activated by both *SPL9 and SPL10* (Wu et al., 2009). Shikata et al. (2009) has found that the first N-terminal portion of *SPL10* encode a functional activator domain which is able to activate transcription in vitro. In rice, *OsSPL14*, an ortholog of *SPL9/15,* have also been proposed to act as a transcriptional activator, with its activation domain located in the C-terminal region (Lu et al., 2013).

Another mechanism for SPL-mediated activation of gene expression is through the recruitment of different *MEDIATOR* complex components, which link transcription factors to RNA Polymerase 2 (Buendía-Monreal and Gillmor, 2016). Hyun et al. (2016) discovered that SPL15 activates the expression of *MIR172* and *FUL* by recruiting *MED18*. Similarly, Yao et al. (2019) showed that SPL10 recruits *MED25* to activate *FUL* and *AP1*.

In Arabidopsis, recent evidence suggest that SPL9/15 may act as a bifunctional transcriptional factor, as SPL9/15 repress axillary meristem formation by directly binding and down-regulating the expression of *LAS* (Tian et al., 2014; Zhang et al., 2020b)

SPLs may also act by directly interacting with other transcription factors and modulating their activity. SPL9 downregulates anthocyanin expression by binding to components of the MYB-bHLH-WD40 activation complex and repressing their transcriptional activity (Gou et al., 2011). It has also been shown that SPL9 repress shoot regenerative capacity by directly binding B-type ARRs and inhibiting their transcriptional activity (Zhang et al., 2015).

1.4.1. miR156-targeted SPLs involved in shoot branching

Overexpression of *MIR156* genes in monocots such as maize, wheat and rice (Chuck et al., 2007; Luo et al., 2012; Liu et al., 2017) and in eudicots such as Arabidopsis, tomato, alfafa and soybean (Schwab et al., 2005; Silva et al., 2014; Aung et al., 2015; Sun et al., 2018) has been associated with an increased branching phenotype, suggesting that some *SPLs* may be associated with bud outgrowth (Fig. 1).

Among the miR156-targeted SPLs, *IDEAL PLANT ARCHITECTURE1* (*IPA1*) which encodes for *OsSPL14*, an ortholog of *SPL9/15*, has been extensively described as a negative regulator of shoot branching. *IPA1 g*ain-of-function mutants in rice show a reduced number of tillers and height while displaying increased panicle branching and grain yield (Jiao et al., 2010; Miura et al., 2010). The negative role of *IPA1* in shoot branching is thought to be mediated by *TB1*, as IPA1 directly binds to the *TB1* promoter to activate its expression and the *tb1* mutant rescues the tillering phenotype of *ipa1* (Lu et al., 2013). The transcriptional activity of IPA1 is regulated by DWARF53 (D53), the rice ortholog of SMXL6/7/8, which directly interact with IPA1 and supress its transcriptional activation activity (Song et al., 2017). In the presence of strigolactones, D53 is targeted by degradation, releasing IPA1 to activate the expression of *TB1* (Song et al., 2017).

The SL-SPL-TB1 pathway is conserved in other species. Liu et al. (2017) have described a similar mechanism in bread wheat, in which TaSPL3 and TaSPL17 repress branching by activating *TB1* expression and their activity is negatively regulated by the interaction with D53. Most recently, a similar mechanism was also described in Arabidopsis, where SMXL6/7/8 disrupts the activation of *BRC1* by SPL9/15 (Xie et al., 2020), although recent evidences suggest that SMXL6/7/8 and SPL9/15 act in parallel pathways (Wang et al., 2020).

UNBRANCHED3 (*UB3*), the maize ortholog of *IPA1*, repress shoot branching through a TB1-independent mechanism (Du et al., 2017). Rice plants overexpressing *UB3*, showed reduced tillering and a global deregulation of cytokinin biosynthesis and signalling genes (Du et al., 2017). While *LOG1* and type-A ARRs were downregulated, OsCKX2, a Cytokinin oxidase/dehydrogenase was highly expressed in the shoot apex of UB3-OE plants, suggesting UB3 is able to control branching by supressing cytokinin metabolism (Du et al., 2017).

Besides *IPA1* orthologs, other SPLs have also been associated with branching inhibition. OsSPL7 repress tillering in rice, at least in part, by inhibiting the expression of *OsGH3.8* which encodes for an enzyme off the GH3 family, responsible for the conjugation of IAA to aspartate, an intermediate of IAA degradation (Dai et al., 2018). AtSPL10 repress branching through the

activation of *AGAMOUS-LIKE MADS BOX PROTEIN 79* (*AGL79*), a putative repressor of bud outgrowth (Gao et al., 2018).

It is possible that several *SPL-like* genes (Fig. 1) were co-opted during evolution to modulate axillary meristem formation and bud outgrowth throughout distinct but interconnected pathways involving auxin, cytokinin and strigolactone signalling.

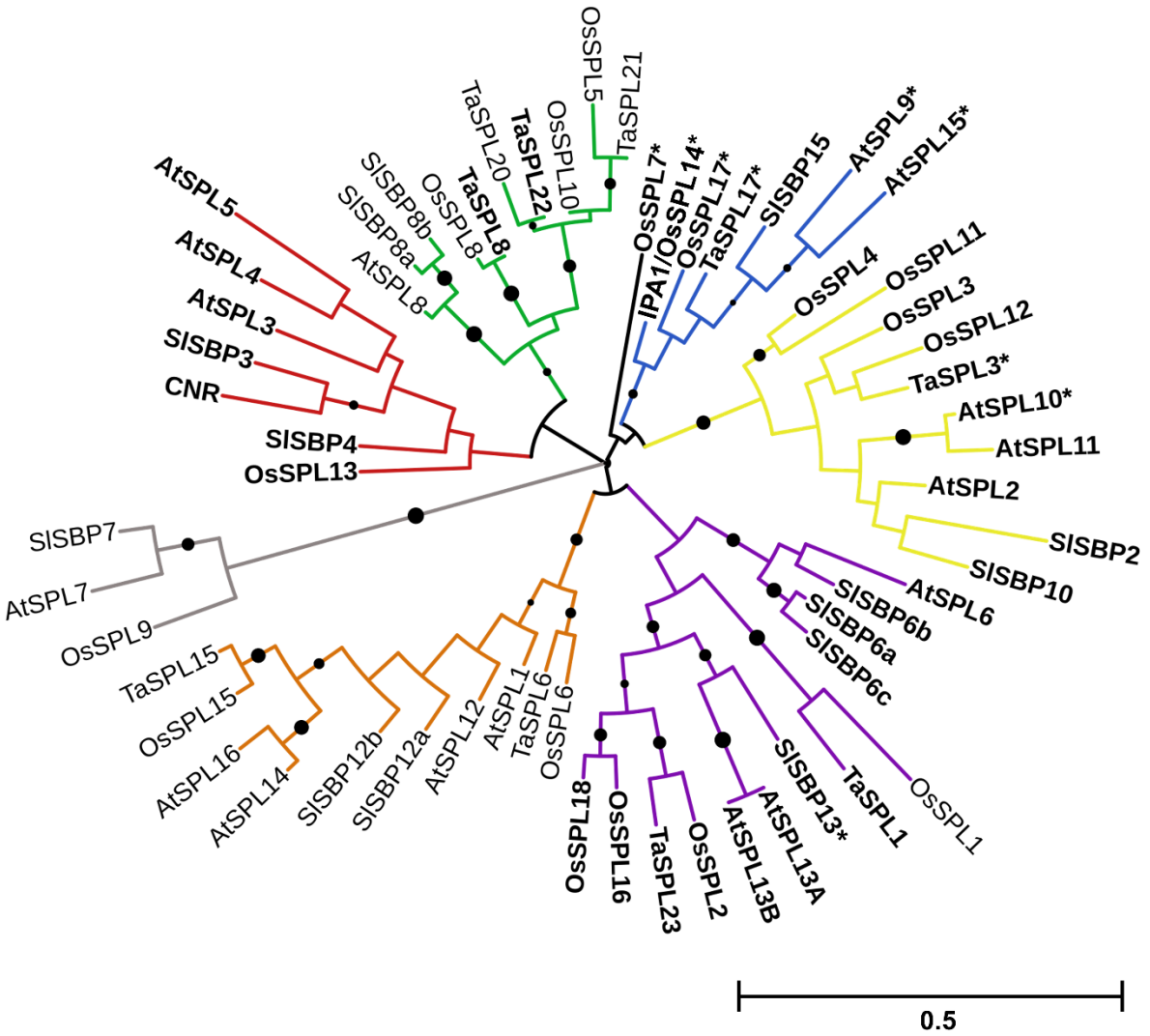

Figure 1. Phylogenetic analysis of SQUAMOSA PROMOTER BINDING PROTEINs in Arabidopsis (AtSPLs), tomato (SlSBPs), rice (OsSPLs) and wheat (TaSPLs). The SBP domains were aligned using ClustalW and the tree was constructed using the maximum likelihood algorithm with 1000 bootstraps. Nodes supported by bootstrap values higher than 50% are marked with a dot, proportional to the bootstrap value. miR156-targeted SPLs are in bold. Different clades are indicated with different colors. Asterisks indicate SPLs previously associated with shoot branching.

1.5. Conclusion

Shoot branching is a key trait for defining the plant shoot architecture. The formation of branches involves great expenditure of resources such as photoassimilates, water and nutrients and have a direct impact on crop yield. The removal of tillers or branches has been associated with an increase in grain or fruit production in wheat, barley and tomato (Alaoui et al., 1988; Kemp and Whingwiri, 1980; Gianfagna et al., 1998; Logendra et al., 2004). Given that the manual removal of side shoots is a labour-intensive and expensive activity for farmers, it is imperative to understand the genetic and physiological mechanisms controlling shoot branching. For instance, Jiao et al. (2010) showed that the low tillering observed in the *ipa1* mutant had a significant increase in rice grain yield in a field trial.

With the emergence of new gene editing techniques and its expansion to non-model crops, shoot branching regulators such as *IPA1* and its orthologs should be further explored as targets for the genetic improvement of crop yield through the modulation of shoot architecture.

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2. THE MICRORNA156-TARGETED *SlSBP15* INHIBITS SHOOT BRANCHING BY MODULATING AUXIN AND CYTOKININ SIGNALLING

ABSTRACT

The formation of branches is an essential aspect of plant development. It initiates with the formation of an axillary meristem in the axil of each leaf. This axillary meristem initiates an axillary bud, composed of a few unexpanded leaves. Depending on endogenous and environmental conditions, axillary bus may remain inhibited or elongate to form a lateral branch. We have previously shown that overexpression of microRNA156 (miR156) in tomato increases the formation of branches. Here we show that the increased branching of miR156 plants is mainly caused by a reduced abundance of *SQUAMOSA PROMOTER BINDING PROTEIN 15* (*SlSBP15*). Plants overexpressing a miR156-resistant version of *SlSBP15*, show inhibited axillary buds throughout development. We show that SlSBP15 regulates axillary meristem formation by inhibiting the expression of *LATERAL SUPPRESSOR*. We also demonstrate that the miR156/SBP module acts by regulating auxin transport and cytokinin biosynthesis. Importantly, we show that, unlike in monocots, SlSBP15 does not activate *SlBRC1b* expression, but rather SlSBP15 interacts with SlBRC1b at the protein-level. Together, these results provide a new mechanism for the regulation of axillary bud activity by the miR156/SBP module.

2.1. Introduction

Plants are highly plastic organisms which are able to modulate their architecture according to their developmental program or in response to environmental conditions. The shoot architecture depends on the activity of meristems, which are groups of undifferentiated cells formed during embryogenesis. While the root apical meristem will form the root system, the shoot apical meristem (SAM) give rise to all shoot organs, including secondary meristems formed in the axils of each leaf, the axillary meristems (AMs), which bear the same developmental potential as the primary SAM. (Domagalska and Leyser, 2011).

The formation of axillary meristems is closely associated with the formation of boundaries, as they emerge in the boundary between the SAM and the leaf primordia. This process is tightly controlled by several the transcription factors such as *LATERAL SUPPRESSOR* (*LS* in tomato, *LAS* in Arabidopsis; Schumacher et al., 1999; Greb et al., 2003) and *GOBLET* (*GOB;* Berger et al., 2009; Rossmann et al., 2015) whose loss-of-function mutants lack axillary meristems during vegetative development.

Once axillary meristems are established, they initiate a few leaves, forming a structure known as axillary bud (AB). ABs may either remain inhibited or outgrow to form a branch, depending on endogenous and environmental signals. While initial bud outgrowth seems be

induced by photoassimilates such as sucrose (Mason et al., 2014; Barbier et al., 2015), sustained bud outgrowth is regulated by a complex network of phytohormones including auxin and strigolactones as repressors and cytokinin as a promoter of AB outgrowth.

Auxin derived from the shoot apex are transported down the stem through the polar auxin transport stream (PATS), mediated primary by the auxin efflux proteins PIN-FORMED (PIN) and ABCB (Gälweiler et al., 1998; Noh et al., 2001). In order to start elongating, ABs need to export auxin, and this is limited by the strength of the bud as an auxin source and the sink strength of the PATS in the main stem (Li and Bangerth, 1999; Balla et al., 2011). Inhibiting the PATS either by decapitating the shoot apex or by treating the stem with an auxin transport inhibitor induces bud outgrowth (Domagalska and Leyser, 2011).

Citokinins promote axillary bud elongation in a dose-dependent manner, either when supplied directly to the buds or to the stem (Wickson and Thimann, 1958; Dun et al., 2012; Roman et al., 2016). Accordingly, in tobacco and tomato, overexpression of cytokinin biosynthesis genes is associated with increased branching (Smigocki, 1991; Eviatar-Ribak et al., 2013).

The family of transcription factors SQUAMOSA PROMOTER-BINDING PROTEINS (SPL in Arabidopsis, SBP in tomato) has been associated with several roles in plant developments, including phase transition, leaf patterning and root and shoot branching (Wang and Wang, 2015). Some members of this family are targeted by microRNA156 (miR156), forming the miR156/SPL module. Overexpression of miR156 has been associated with increased shoot branching in several species such as maize, wheat, rice, Arabidopsis and tomato (Chuck et al., 2007; Luo et al., 2012; Liu et al., 2017; Schwab et al., 2005; Silva et al., 2014). The rice *IDEAL PLANT ARCHITECTURE1* (*IPA1*) gene, which encodes for *OsSPL14*, is thought to counteract miR156 in shoot branching, as *IPA1* gain-of-function mutants show reduced tillering in rice (Miura et al., 2010; Jiao et al., 2010). *IPA1* orthologs in other species such as TaSPL17 in wheat (Liu et al., 2017) and SPL9 and SPL15 in Arabidopsis (Schwarz et al., 2008) are also associated with the repression of shoot branching. The TCP transcription factor *TEOSINTE BRANCHED 1* (*TB1* in monocots, *BRC1* in dicots) is a major repressor of axillary bud activity (Hubbard et al., 2002; Takeda et al., 2003; Aguilar-Martinez et al., 2007; Martín-Trillo et al., 2011). In rice, wheat and Arabidopsis *IPA1* orthologs have been shown to suppress branching by directly activating the expression of *TB1/BRC1* (Lu et al., 2013; Liu et al., 2017; Xie et al., 2020). Other SPLs, such as maize *UNBRANCHED3* and rice *OsSPL7* have been shown to supress branching by modulating cytokinin biosynthesis and auxin degradation, respectively (Du et al., 2017; Dai et al., 2018).

Here we show that the miR156/SBP module controls multiple aspects of the shoot branching in tomato, from the establishment of the AMs to bud outgrowth. Overexpression of

miR156 in tomato increases shoot branching and this effect is counteracted by the tomato ortholog of *IPA1*, *SlSBP15*, which when overexpressed inhibits axillary bud outgrowth. MiR156-targeted SlSBP15 directly represses *LS* expression, but this is not sufficient to inhibit AM formation in *SlSBP15*-overexpressing plants. We found that the miR156/SBP module regulate tomato shoot branching by modulating auxin transport and cytokinin biosynthesis. We also show that SlSBP15 does not activate *SlBRC1b* expression, but SlSBP15 can interact with SlBRC1b at the protein level. Taken together, these results may provide a novel mechanism by which shoot branching is regulated by miR156-targeted *SPLs*.

2.2. Results

2.2.1. Overexpression of microRNA156 is associated with increased shoot branching

Although the role of the miR156/SPL module in shoot branching has been documented in several species, the underlying molecular mechanisms are poorly understood. To initially understand how the miR156/SPL module controls vegetative architecture in tomato at the molecular level, we assessed shoot branching in tomato plants overexpressing microRNA156 (*p35S:MIR156b*, hereafter referred as 156OE). These plants show a dramatic change in shoot architecture, with an increased shoot branching phenotype (Fig. 1A). In tomato (cv. Micro-Tom or MT), axillary buds usually remain dormant during vegetative development. After flowering transition, the two axillary buds below the inflorescence always elongate into shoots. One will form the sympodial shoot, which will take the place of the apical meristem, and the axillary bud right below will form a new branch (Fig. 1 B, 1D-E). Occasionally, other branches are also formed. In 156OE plants, buds start to outgrow during vegetative phase, and after flowering almost every AB will elongate into a vigorous branch (Fig. 1C-E).

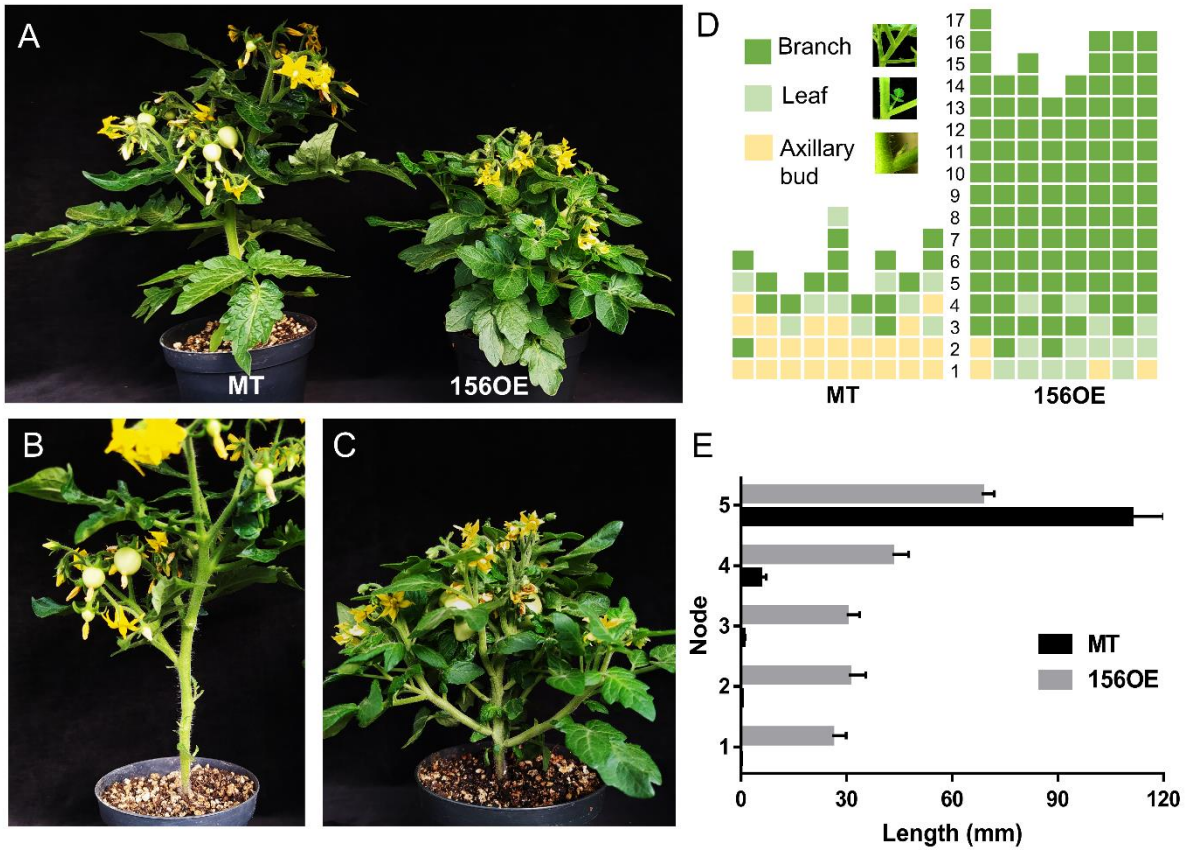

Figure 1. Overexpression of microRNA156 increases shoot branching in tomato. **A.** Representative MT and 156OE plants at 35 days after germination (DAG). **B-C.** Same plants as in A, with leaves below the inflorescence detached. **D.** Branching pattern scheme of MT and 156OE plants. Every column represents a different plant, while every line indicates the status of each leaf axil. **E.** Length of the axillary shoots in the first five nodes of MT and 156OE plants. Node 1 is the first node above the cotyledons.

2.2.2. miR156-targeted *SlSBP15* is the major contributor for shoot branching inhibition

In tomato, 15 *SlSBPs* have been described thus far, of which 10 are reported to be regulated by the miR156. *SlSBP15* is the tomato homolog of *IPA1* (see previous chapter, Fig. 1) which has been associated with reduced tillering in rice (Jiao et al., 2010; Miura et al., 2010). Therefore, we investigated whether *SlSBP15* had any effect in shoot branching, by assessing the phenotypes of rSBP15OE plants which overexpress the *SlSBP15* ORF with synonymous mutations in the miR156 binding site (described in Corrêa, 2019). rSBP15OE plants show inhibited branching throughout plant development (Fig. 2) and their axillary buds are usually smaller than MT buds and sometimes even not visible to the naked eye (Fig. S1). In 156OE plants, several miR156-targeted *SlSBPs* are downregulated (Silva et al., 2014). To assess whether *SlSBP15* is the major *SlSBP* associated with shoot branching, we generated double transgenic rSBP15OE;156OE plants. In a 156OE background, the expression of *rSBP15* was sufficient to completely suppress

branching (Fig. 2C), suggesting *SlSBP15* is fundamental to inhibit shoot branching in tomato. We have also assessed the roles of other *SlSBPs* in shoot branching. Tomato MT plants overexpressing a miR156-resistant version of *SlSBP3* (rSBP3OE; Corrêa, 2019) also show suppressed branching throughout vegetative development (Fig. S2). Unlike rSBP15OE plants, axillary buds in rSBP3OE have a similar size as in MT plants, but only rarely elongate into a branch (Fig. S2C-D). However, when crossed to a 156OE plants, rSBP3OE fail to suppress branching, suggesting that *SlSBP3* has a minor role in the control of bud outgrowth (data not shown).

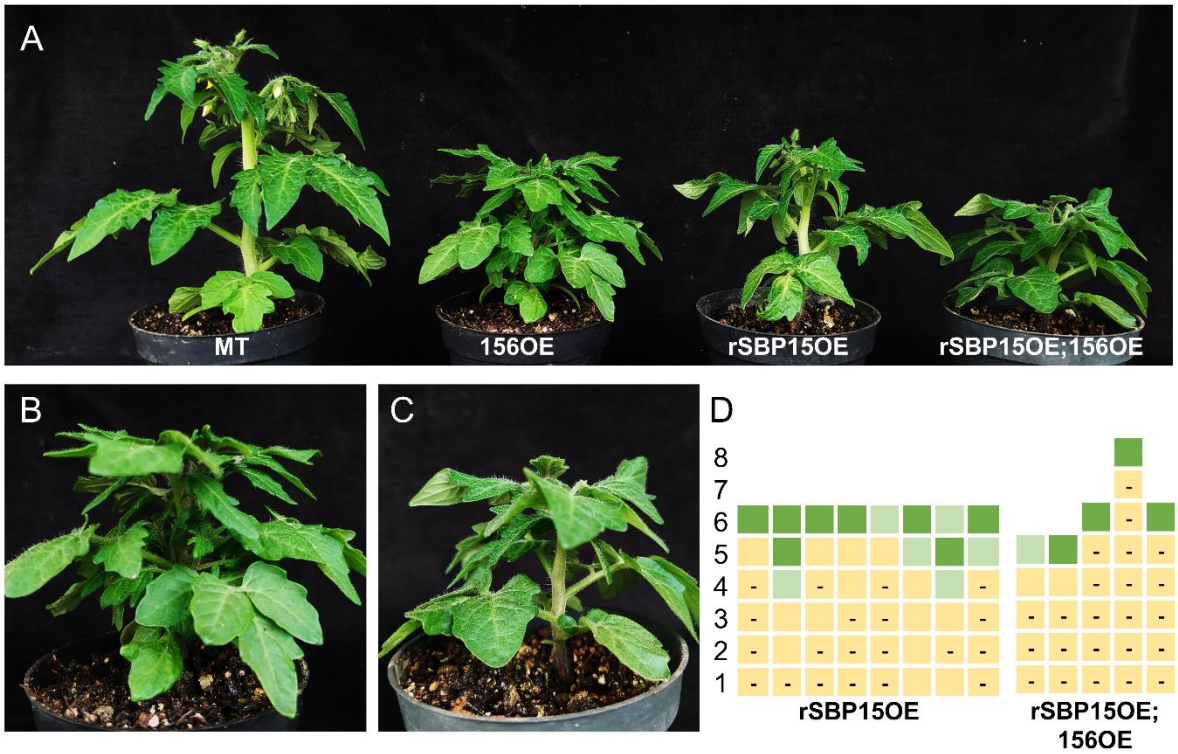

Figure 2. miR156-targeted *SlSBP15* is the major *SlSBP* contributing to the control of shoot branching. **A.** Representative MT, 156OE, rSBP15OE and rSBP156OE; 156OE plants at 30 DAG. **B.** 156OE and **C.** rSBP15OE plants shown in A. **D.** Branching pattern scheme of rSBP15OE and rSBP15OE; 156OE plants. Every column represents a different plant, while every line indicates the status of each leaf axil. A yellow box with a minus sign denotes an inhibited axillary bud.

To examine the branching phenotypes caused by miR156 and *SlSBP15* overexpression in a non-dwarf tomato cultivar, we have crossed 156OE and rSBP15OE with wild-type tomato cv. Moneymaker and analysed the F1 offspring, in which the known recessive mutations of Micro-tom are in heterozygosity (Martí et al., 2006). Overexpression of miR156 in the hybrid Moneymaker/MT F1 offspring caused the same highly branched phenotype as observed in MT, with vigorous branches forded from almost every leaf axil. Axillary buds of rSBP15OE in the hybrid background were bigger than in MT background, but never formed an axillary branch (Fig.

S3). This finding indicates that the higher expression of *SlSBP15* blocks bud outgrowth in a hybrid background as well.

2.2.3. SBP15 regulates the *LATERAL SUPPRESSOR*

Given that the axillary buds of rSBP15OE are smaller in comparison to the wild-type (MT) and sometimes even not visible, we investigated whether *SlSBP15* inhibit branching through affecting the axillary meristem formation pathway controlled by *LATERAL SUPPRESSOR* (*LS*). We initially generated double 156OE; *ls* plants by crossing 156OE plants with the *ls* mutant, which completely abolished the high branching phenotype observed in 156OE plants (Fig. 3A-B). This observation indicates that *LS* is essential for the formation of branches in 156OE plants . We then tested whether *SlSBP15* was able to negatively regulate *LS* expression. We identified several putative SPL/SBP binding sites (GTAC) in the ~1 Kb promoter of *LS* (Fig. 3C). Thus, we generated constructs containing the firefly *luciferase* gene (*LUC*) under the control of the *LS* promoter (*pLS:LUC*) and co-expressed it in *Nicotiana benthamiana* leaves in the presence of either the *p35S:rSBP15* construct or an empty vector. *SlSBP15* was able to reduce *pLS:LUC* expression in *N. benthamiana* leaves (Fig. 3D). We measured the expression of *LS* in floral buds, a tissue enriched in boundary cells (Tian et al., 2014). Accordingly, *LS* expression was downregulated in rSBP15OE buds (Fig. 3E). These results suggest that *LS* is downstream of the miR156/SPL node and SlSBP15 is able to directly regulate *LS* expression.

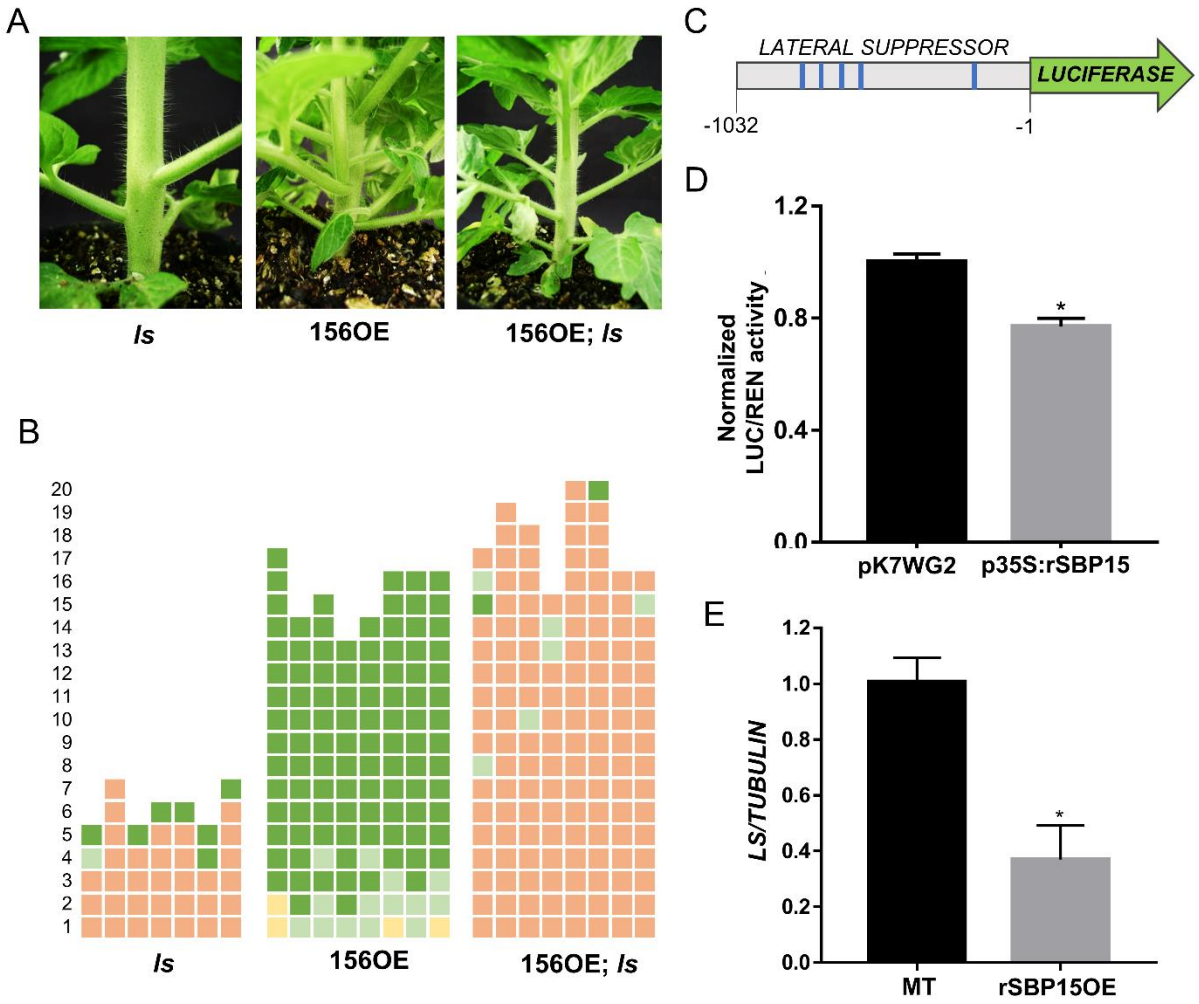

Figure 3. *LS* is downstream of the miR156/SPL module. **A.** Representative *ls*, 156OE and 156OE; *ls* plants at 30 DAG. **B.** Branching pattern scheme of the genotypes shown in A. **C.** DNA construct used for dual-luciferase assay, showing the cloned *LS* promoter fused to Luciferase. Putative SPL/SBP binding sites are shown as vertical blue lines. **D.** Dual-luciferase assay of pLS:LUC co-expressed in *N. benthamiana* leaves either with the p35S:rSBP15 construct or the empty vector (pK7WG2). The luminescence of *Renilla Luciferase* (REN) expressed under the 35S promoter, was used as an internal control. The relative activity of the *LS* promoter is expressed as a ratio of *Firefly Luciferase* (LUC) and REN. Values are mean ± SD of three biological replicates. p-value < 0.05. **(E)** Relative expression of *LS* in floral buds of MT and rSBP15OE plants. Values are mean ± SE of three biological replicates. p-value < 0.05.

2.2.4. Axillary buds in rSBP15OE plants are functional

Provided that SlSBP15 negatively regulates the expression of *LS* (Fig. 3), which is an essential gene in the formation of axillary meristems and axillary buds in rSBP15OE are sometimes non-visible, we hypothesized that axillary buds in rSBP15OE plants had defective axillary meristems and thus, were unable to form axillary branches. Decapitation is a treatment known to trigger axillary bud outgrowth by removing the inhibition from auxin derived from the shoot apex (Thimann and Skoog, 1933). We then decapitated MT, 156OE and rSBP15OE plants at 30 DAG and measured the outgrowth of axillary buds in nodes 1, 2 and 3 at 7 and 14 days after decapitation

(Fig. 4). All axillary buds of MT, 156OE and, to our surprise, rSBP15OE plants, gave rise to axillary branches in response to decapitation (Fig. 4A-C). Seven days after decapitation, the size of axillary buds at node 3 increased 135% and 68% in MT and 156OE plants, respectively. Surprisingly, at the same time, buds in decapitated rSBP15OE increased 800% in comparison to intact plants (Fig. 4D). Fourteen days after decapitation, the most apical branch of MT plants usually dominated over the others, while in 156OE plants, all branches grew almost evenly (Fig 4E). Interestingly, in rSBP15OE, the dominance from the most apical branch was even stronger than in MT, growing almost 3 times more than the lower branches. (Fig. 4E). Together, these results suggest that rSBP15OE plants have active axillary meristems which are under a strong inhibition and decapitation is able to release this inhibition. Additionally, overexpression of the miR156-resistant version of *SlSBP15* seems to increase the competition between buds as seen by the dominance of the most apical bud over the others.

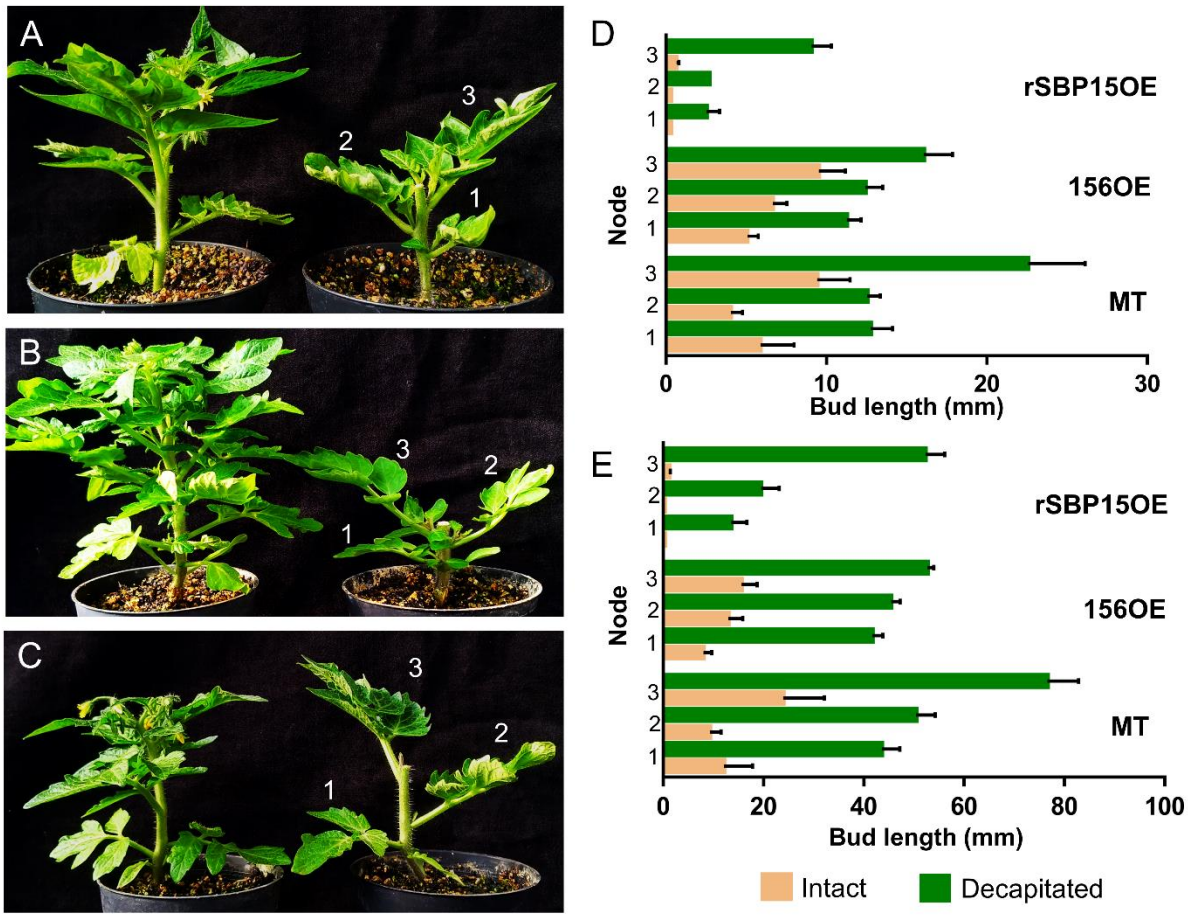

Figure 4. Decapitation trigger axillary bud outgrowth in MT, 156OE and rSBP15OE plants. Representative intact and decapitated MT **(A)**, 156OE **(B)** and rSBP15OE **(C)** at 7 days after decapitation. After decapitation, branches were measured in intact and decapitated plants after 7 **(D)** and 14 days **(E)**.

2.2.5. The miR156/SPL module regulates auxin transport

Given that decapitation is able to release axillary bud dormancy even in rSBP15OE plants, we hypothesized that polar auxin transport through the PATS in the stem intersects with the miR156/SBP module to control bud outgrowth. PATS is known to inhibit the export of auxin from axillary buds, consequently inhibiting their outgrowth ((Domagalska and Leyser, 2011). We examined whether the high and low branching phenotypes observed in 156OE and rSBP15OE plants (Fig. 1 and 2), respectively, was due to the modulation of auxin transport via PATS. We measured the basipetal transport of [^{3}H]IAA (Indole-3-acetic acid) applied to 10-mm hypocotyl sections of MT, 156OE, rSBP15OE, as well as the negative control treated with 1-Naphthylphthalamic acid (NPA), a well-known PATS inhibitor (Bennett et al., 2006). While 156OE seedlings showed a significant reduction in the auxin transport rate, rSBP15OE seedlings showed only a slight increase in IAA transport, although not significant (Fig. 5A). It is possible that the de-repression of other *SlSBPs* is necessary to affect auxin polar transport in the seedling developmental stage. As the bulk of auxin transport in the stem is mediated by PIN1 protein, we examined whether the reduced auxin transport in 156OE plants was due to the transcriptional regulation of *SlPIN1* levels. We measured *SlPIN1* expression in hypocotyls through qRT-PCR and found that *SlPIN1* mRNA levels were reduced in 156OE and upregulated in rSBP15OE hypocotyls, though not significantly (Fig. 5B). Likewise, *SlPIN4* levels are significantly decreased in 156OE hypocotyls but only slightly increased in rSBP15OE hypocotyls (Fig. 5C).

We also took advantage of plants harbouring the *pAtPIN1:AtPIN1-GFP* (*PIN1-GFP*) construct in MT and 156OE backgrounds to assess AtPIN1 expression and localization in hypocotyls. We could observe that in 156OE seedlings, PIN1-GFP signal was significantly lower in basal plasma membranes of the xylem parenchyma (Fig. 5 D-E).Together, these results suggest that 156OE plants have decreased auxin transport in the stem, and this is probably due to a down-regulation of *SlPIN* proteins, although it is not clear whether SlSBP15 alone or together with other SlSBPs directly regulate the expression of *SlPIN* genes.

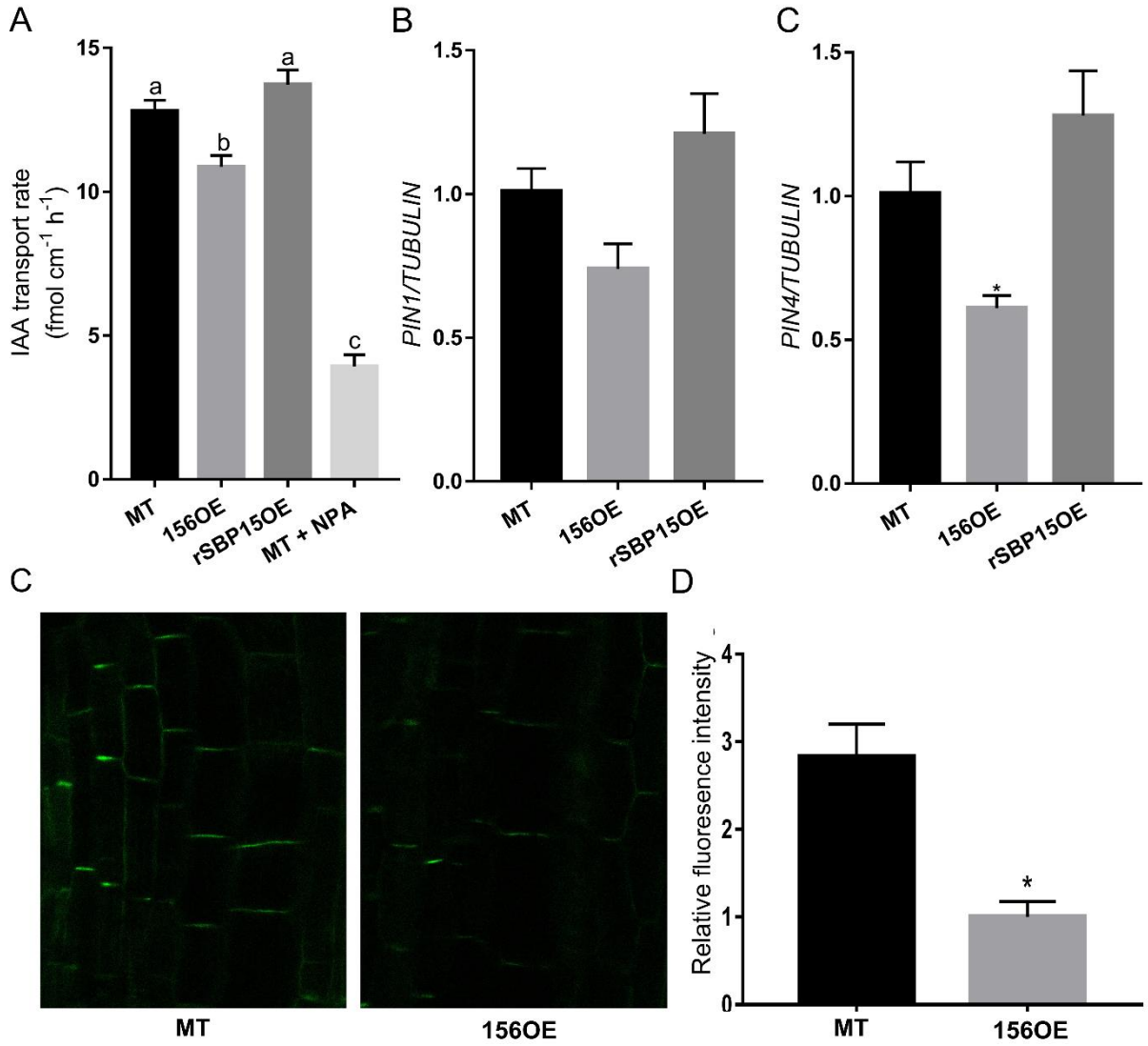

Figure 5. The miR156/SBP module regulates auxin transport in tomato seedlings. **A.** Basipetal [^{3}H]IAA transport is shown in 10-mm hypocotyl sections of the 2-week-old MT,156OE, rSBP15OE and MT seedlings treated with 1-Naphthylphthalamic acid (NPA) as a negative control. Values are mean ± SE (n = 16). Letters represent significant differences defined through an ANOVA followed by Tukey's multiple comparisons tests (p-value < 0.05). *SlPIN1* (**B**) and SlPIN4 (**C**) mRNA levels in hypocotyl sections of 2-week-old seedlings measured by qRT-PCR (n=4). **D.** Confocal microscopy images of *pAtPIN1:AtPIN1-GFP* in the MT and 156OE backgrounds. **E.** Quantification of GFP fluorescence intensity in the same plants shown in C. Values are the mean of two biological replicates ± SE. Asterisk represents significance at p-value<0.05.

2.2.6. miR156/SBP module regulates cytokinin biosynthesis

Experiments in pea have shown that auxin in the main stem inhibits the expression of ADENYLATE ISOPENTENYLTRANSFERASE1 (PsIPT1) and PsIPT2 (Tanaka et al., 2006), which encode for enzymes that catalyse the first step of cytokinin biosynthesis. Therefore, we assessed whether the low basipetal auxin transport in 156OE was able to affect cytokinin biosynthesis. We measured the expression of SlIPT3 and SlIPT4, which are prominently expressed

in tomato stems (Matsuo et al., 2012). Surprisingly, the hypocotyls of 156OE plants were depleted of both SlIPT3 and SlIPT4 (Fig. 6A-B), however the expression of SlIPT4 was induced in axillary buds of these plants.

SlIPT3 expression was significantly up-regulated in both hypocotyls and axillary buds of rSBP15OE while IPT4 expression was unaffected by overexpression of SlSBP15 in either tissue (Fig. 6A-D). These results suggest that, unexpectedly, SlSBP15 activates SlIPT3 expression while SlIPT4 is specifically induced in axillary buds but not in hypocotyls of 156OE plants, suggesting a complex tissue-specific regulation of cytokinin biosynthesis by the miR156/SBP module, although SlSBP15 does not seem to regulate SlIPT4.

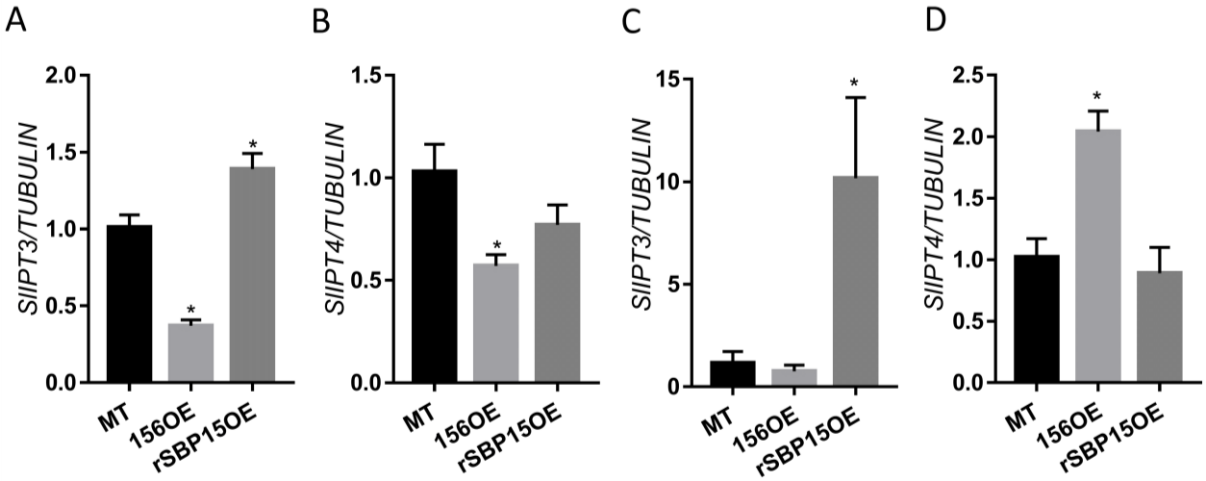

Figure 6. The miR156/SBP module regulates cytokinin biosynthesis. **A-B.** Expression of *SlIPT3* and *SlIPT4* in hypocotyl sections of 2-week-old tomato. **C-D.** Expression of *SlIPT3* and *SlIPT4* in axillary buds of 30-day-old tomato plants. Gene expression was measured by qRT-PCR. Values are mean ± SE of four biological replicates. Asterisks represent statistical significance at p-value<0.05.

2.2.7. SlSBP15 controls *SlBRC1b* at the transcriptional level

Orthologs of *SlSBP15* in wheat, rice and Arabidopsis (Fig. 1, previous chapter) inhibit shoot branching by activating the expression of *TB1*-like genes (Lu et al., 2013; Liu et al., 2017; Xie et al., 2020). We therefore tested whether *SlSBP15* could control *TB1*-like genes in tomato axillary buds. Two *TB1-like* genes have been described in tomato, *BRANCHED1a* (*SlBRC1a*) and *SlBRC1b*, of which *SlBRC1b* seems to have a major role in the inhibition of shoot branching (Martín-Trillo et al., 2011). We measured the expression of *SlBRC1a* and *SlBRC1b* in axillary buds of MT, 156OE and rSBP15OE plants. Surprisingly, we detected a higher expression of *SlBRC1b* in axillary buds of 156OE, and both *SlBRC1a* and *SlBRC1b* were downregulated in rSBP15OE buds (Fig. 7A-B). To assess whether this was a direct effect of *SlSBP15*, we checked the promoter region of *SlBRC1b* and identified several putative SPL/SBP binding sites (Fig. 7C). We then

performed a luciferase transactivation assay in which we co-expressed a *pBRC1b:LUC* construct with either *p35S:rSBP15* construct or *p35S:NLS-GFP* as a negative control. According to this assay, in the presence of rSBP15, *LUC* expression dramatically decreased, suggesting a direct regulation of *SlBRC1b* by SlSBP15 (Fig. 7D).

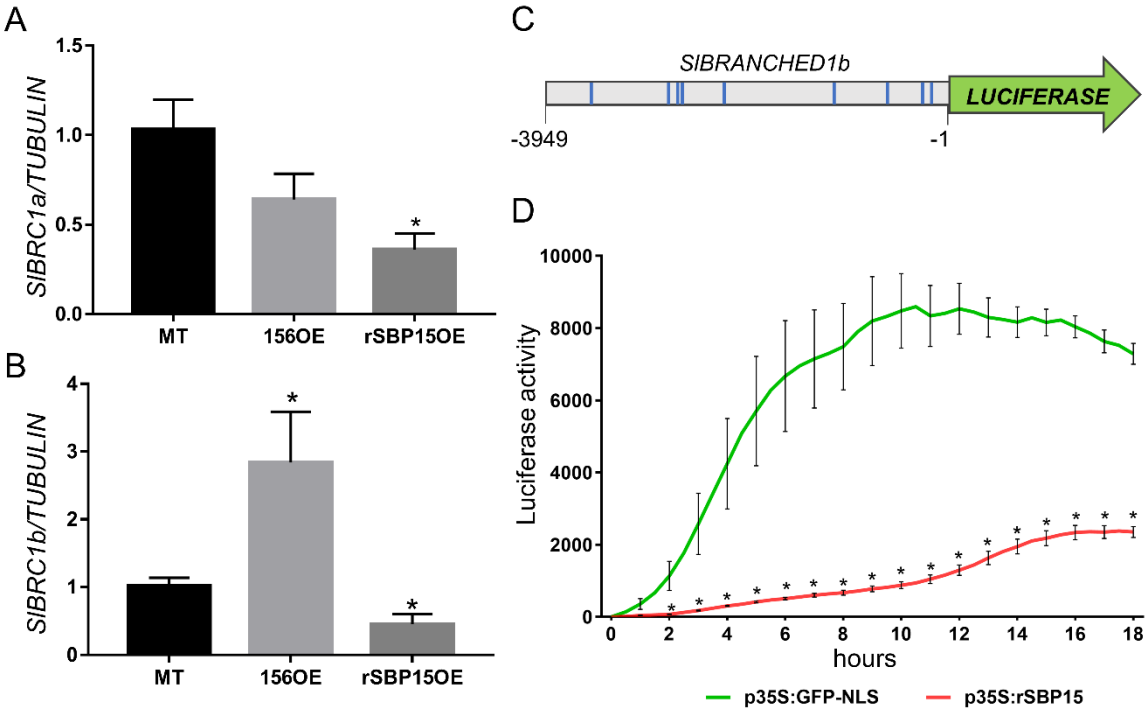

Figure 7. SlSBP15 regulates *SlBRANCHED1b* at the transcriptional level. Relative expression of *SlBRC1a* **(A)** and *SlBRC1b* **(B)** in axillary buds of MT, 156OE and rSBP15OE plants. Values are mean ± SE of four biological replicates. Asterisks represent statistical significance (p-value<0.05) **C.** Schematic representation of the *SlBRC1b* promoter used in the luciferase transactivation assay. Blue lines indicate putative SPL/SBP binding sites. **D.** Luciferase transactivation assay of *pBRC1b:LUC* co-expressed with *p35S:rSBP15*. A *p35S:NLS-GFP* construct was used as a negative control. Values represent mean relative luminescence units of five biological replicates ± SEM, measured every 30 minutes, during 18 hours. Asterisks represent statistical significance at p-value<0.05.

2.2.8. SlSBP15 and SlBRC1b interact at the protein level

As the transcriptional repression of *SlBRC1b* expression did not reflect the inhibited branching phenotype of rSBP15OE plants, we hypothesized that the miR156-targeted SlSBPs could possibly interact with the SlBRC1 pathway through a protein-protein interaction. To test this hypothesis, we initially performed a yeast-two-hybrid (Y2H) assay. SlSBP15 and SlSBP3 ORFs were fused to the Gal4-binding domain (BD) and co-transformed in yeast either with an empty vector or with SlBRC1a or SlBRC1b ORFs fused to the Gal4-activation domain (AD). We observed that SlSBP15 was able to interact with SlBRC1b but not with SlBRC1a, as shown by yeast

growth in media lacking Leu, Trp and His in the presence of 3-amino-1,2,4-triazole (3-AT) (Fig. 8). Interestingly, we could also observe a weak interaction between SlSBP3 and SlBRC1b.

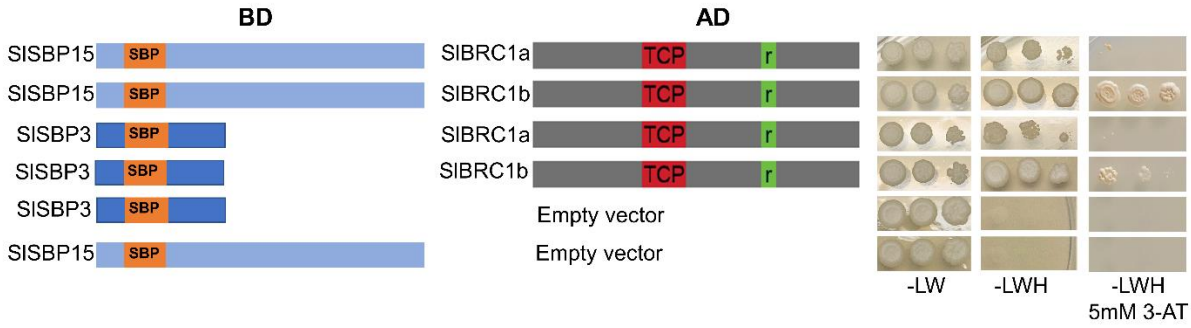

Figure 8. SlSBP15 and SlBRC1b interact in a yeast-two-hybrid assay. SlSBP15 and SlSBP3 ORFs were cloned into pGBKT7-BD vector while SlBRC1a and SlBRC1b ORFs (including the TCP and R domain, putatively involved in protein interactions; Cubas et al., 1999) were cloned in pGADT7-AD. Vectors were co-transformed in the yeast strain AH109. Transformants were selected in media lacking leucine and tryptophan (-LH) and assayed for interaction in media lacking leucine, tryptophan and histidine (-LWH) in the presence or absence of 5mM of 3-amino-1,2,4-triazole (3-AT). Each row shows ten-fold serial dilutions of the indicated strain.

2.3. Discussion

The formation of axillary branches demands energy and valuable resources from plants, which could be instead directed to the formation of fruits. In tomato production, branches are commonly called "suckers" and their removal is associated with the production of larger fruits in a shorter period of time (Ara et al., 2007). Therefore, understanding how branching is regulated is of great value for engineering plants for increased productivity.

The miR156/SBP module has a conserved role in shoot branching. Overexpression of miR156 is associated with a loss of apical dominance in both monocots and eudicots. Here we report that high levels of the microRNA156 substantially increases branching in tomato. In 156OE plants, axillary branches initiate much earlier than in wild-type tomato plants. After flowering, tomato plants usually initiate branches in the two axils below the inflorescence, whereas in 156OE plants almost all axils develop into branches. It must be noted, however, that the increased branching pattern in 156OE plants is accompanied by pleiotropic effects, such as delayed flowering time, as seen by the increased number of leaves, and abnormal flower and fruit morphology as we have reported before (Silva et al., 2014, 2019).

In tomato, 10 out of 16 *SBPs* are regulated by the miR156 (Salinas et al., 2012). Here we show, that, at least miR156-targeted *SlSBP15* is fundamental for controlling shoot branching, as its overexpression is sufficient for reverting the highly shoot branching phenotype of 156OE to nearly

wild-type levels. Additionally, SlSBP15 also counteract miR156 overexpression effects on flowering time, as shown by the number of leaves produced until flowering (Fig. 2), showing that a single *SBP* may control branching and flowering time.

In Arabidopsis, it has been shown that SPL9 and SPL15 negatively regulate *LAS* expression (Tian et al., 2014), a gene essential for the formation of axillary meristems. Similarly, tomato *LS* expression is negatively modulated by SlSBP15 (Fig. 3). However, plants overexpressing *SlSBP15* still produce functional axillary meristems (Fig. 4) as seen in Arabidopsis, where plants expressing a miR156-resistant version of *SPL9* also show normal axillary meristems. It must me noted, however, that rSBP15OE axillary buds are much smaller than their wild-type counterparts (Fig. S1). We have previously shown that *SlSBP15* regulates floral meristem size in tomato (Corrêa, 2019). Therefore, instead of blocking axillary meristem formation, *SlSBP15* may regulate their size by down-regulating *LS* and perhaps other meristem size-associated genes such as *WUSCHEL* (*WUS*) and *WUS-like* genes. As a support for this hypothesis, it has been recently shown that the miR156/SPL module in Arabidopsis regulates SAM size via *WUS* expression independently of the *CLAVATA3* (*CLV3*)-*CLV1* pathway (Fouracre and Poethig, 2019).

Auxin is mainly produced by the growing leaves of the shoot apex and is transported downstream mostly through the PATS. In order to outgrow, ABs need to export auxin (Li and Bangerth, 1999). PATS auxin sink competes with the apical bud in terms of auxin transport (Balla et al., 2011). Reducing auxin transport in the PATS either by removing the apex or by treating stems with an auxin transport inhibitor induces bud outgrowth. 156OE plants have reduced auxin transport in the stem, and this is probably caused by downregulation of auxin efflux proteins. We show that *SlPIN1* and mostly *SlPIN4* are down-regulated in 156OE and up-regulated in rSBP15OE hypocotyls (Fig. 5). In rice, IPA1/OsSPL14 directly binds to the promoter of *OsPIN1b* (Lu et al., 2013). It remains to be demonstrated whether in tomato this regulation is direct or not.

Auxins antagonize cytokinin biosynthesis by repressing the expression of *IPT* genes in the stem (Tanaka et al., 2006). Here we demonstrated that, unexpectedly, *SlIPT3* expression was induced by overexpression of *SlSBP15* both in hypocotyls and axillary buds, while being less abundant in the hypocotyls of 156OE seedlings (Fig. 6) We also showed that *SlIPT4* is upregulated in axillary buds and depleted in hypocotyls of 156OE plants, suggesting a tissue-specific regulation, although this does not seem to be mediated by *SlSBP15* (Fig. 6). It must be noted that for the expression analysis, hypocotyls were collected from 2-week-old tomato seedlings, before axillary bud outgrowth had started. It would be interesting to check the expression of CK biosynthesis genes in the stems of older tomato plants.

IPA1 and its orthologs in wheat and Arabidopsis have been hypothesized to be upstream of the TCP transcription factor *TB1/BRC1*, by directly activating its expression (Lu et al., 2013; Li et al., 2017; Xie et al., 2020). Here we showed that SlSBP15 role in branching inhibition is not mediated by *SlBRC1* activation, as we show that high levels of *SlSBP15* leads to the repression of *SlBRC1b* expression (Fig. 7). However, SlSBP15 and SlBRC1b can interact at the protein level. Interactions between SPLs and members of the TCP family have already been described. In Arabidopsis, SPL9 interacts with TCP4 (Rubio-Somoza et al., 2014). In rice, Lu et al. (2013) demonstrated that IPA1 can interact with PCF1 and PCF1, while also showing a weak interaction between IPA1 and TB1. As both *SlSBP15* and *SlBRC1b* seem to inhibit tomato shoot branching, it remains to be determined whether their interaction may result in a cooperative regulation of common downstream targets during axillary bud outgrowth. To initiate this analysis, we are currently evaluating RNA-seq data from axillary buds harvested from MT, 156OE and rSBP15OE plants in order to identify possible SlSBP15 targets in tomato in a genome-wide fashion.

2.4. Methods

2.4.1. Plant material and growth conditions

Tomato plants (*Solanum lycopersicum*) were grown in 1:1 mixture of commercial substrate and vermiculite, under standard greenhouse conditions. Unless stated all plants used in this study were in the *S. lycopersicum* cv. Micro–Tom (MT) background. Plants overexpressing *AtMIR156b* under the control of the 35S promoter were previously described by Silva *et al.* (2014); rSBP15OE and rSBP3OE plants, overexpressing miR156-resistant versions of *SlSBP15* and *SlSBP3* were described by Corrêa (2019). The *lateral suppressor* (*ls*) mutant in the MT background was previously described (Lombardi-Crestana et al., 2012). A *pAtPIN1:AtPIN1-GFP* construct (Bayer et al., 2009) was transformed into MT plants to generate MT; *pAtPIN1:AtPIN1-GFP* transgenic plants.

2.4.2. Gene expression analysis by qRT-PCR

Total RNA was extracted by using TRIzol reagent (Invitrogen) and treated with DNase I (Invitrogen), according to the manufacturer's instructions. RNA was reverse-transcribed with Improm II Reverse Transcriptase (Promega) to generate cDNA. PCR reactions were performed using GoTaq qPCR Master Mix (Promega) in a Step-One-Plus real-time PCR system (Applied Biosystems). For each genotype, three technical samples of three to four biological samples were

analysed, using the tomato gene *TUBULIN* (Solyc04g081490) as an internal control (Expósito-Rodríguez et al., 2008). All primers used are described in Table S1.

2.4.3. Luciferase transactivation assays

A fragment of 1032 bp of the LS promoter was cloned into pGREEN-0800-LUC (Hellens et al., 2005) with BamHI and NcoI to generate the pLS:LUC construct. The effectors p35S:rSBP15 and p35S:rSBP3 in the pK7WG2 vector were previously described (Corrêa, 2019). The reporter and effectors constructs were transformed in Agrobacterium tumefaciens GV3101 electrocompetent cells. Overnight grown cultures were resuspended in agroinfiltration medium (10mM $MgCl_2$, 200µM acetosyringone) at an OD=0.4 and incubated in the dark for 2 hours. pLS:LUC in combination with either p35S:rSBP15 and or the empty pK7WG2 vector were agroinfiltrated in 5-week-old Nicotiana benthamiana leaves. Firefly (LUC) and Renilla (REN) luciferase acitivity were assayed two days after agroinfiltration, using the Dual-Luciferase Assay kit (Promega) in a GloMax Multi+ luminometer (Promega). Two readings of three biological replicates were made. Results were expressed as a LUC/REN ratio and normalized to 1.

For the SlBRC1b promoter, 3949bp upstream of the start codon were cloned by assembling 5 promoter fragments, the LUC ORF and the terminator NOS in a pDGBα2 vector, through a GoldenBraid reaction (Sarrion-Perdigones et al., 2013). Agroinfiltration was performed as above. Leaf disks were incubated with D-Luciferin (Promega) and luminescence was measured every 30 minutes, during 18 hours.

2.4.4. Polar Auxin Transport

Basipetal [^{3}H]IAA transport was measured in 10-mm hypocotyl sections of 2-week-old MT,156OE and rSBP15OE plants as described by Silva et al. (2018). MT treated with NPA was used as a negative control.

2.4.5. Confocal microscopy

Two-week-old tomato plants harbouring the *pAtPIN1:AtPIN1-GFP* construct in the MT or 156OE background were used for imaging. Hypocotyls were longitudinally halved by hand. Sections were immersed in MS medium (1/2X) with low melting point agarose and observed in a *Leica SP8* confocal microscope. Images were taken at the same conditions for both genotypes. Fluorescence intensity was measured at 10 basal plasma membranes for each genotype using

ImageJ. The five most intense plasma membranes were used for calculation of the mean fluorescence.

2.4.6. Yeast-two-hybrid

SlSBP15 and SlSBP3 ORFs were cloned into pGBKT7-BD vector while SlBRC1a and SlBRC1b ORFs were cloned in pGADT7-AD. Yeast strain AH109 was grown overnight in YPDA medium, and competent cells were prepared and BD and AD vectors were co-transformed by using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Gietz and Woods, 2002). Transformants were selected in media lacking leucine and tryptophan (-LH). Strains were assayed for interaction in media lacking leucine, tryptophan and histidine (-LWH) in the presence or absence of 5mM 3-AT. Each row shows ten-fold serial dilutions of the indicated strain.

2.5. SUPPLEMENTARY MATERIAL

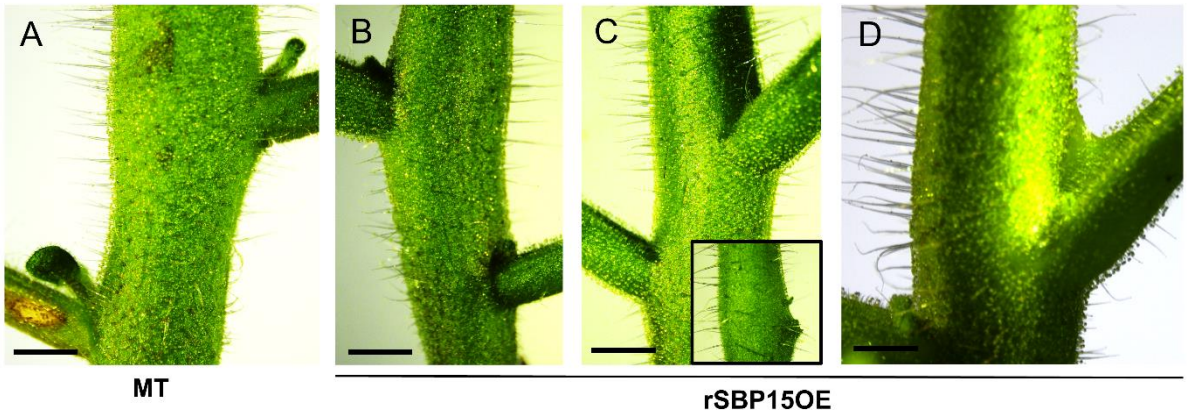

Figure S1. Axillary buds of MT (A) and rSBP15OE (B-D). Axillary buds of rSBP15OE plants are smaller in size (B) and sometimes not visible to the naked eye (C), unless the subtending leave is removed. Ocasionally, in rSBP15OE plans, petioles do not fully separate from the stem (D). Scale bars in A-D represent 2mm.

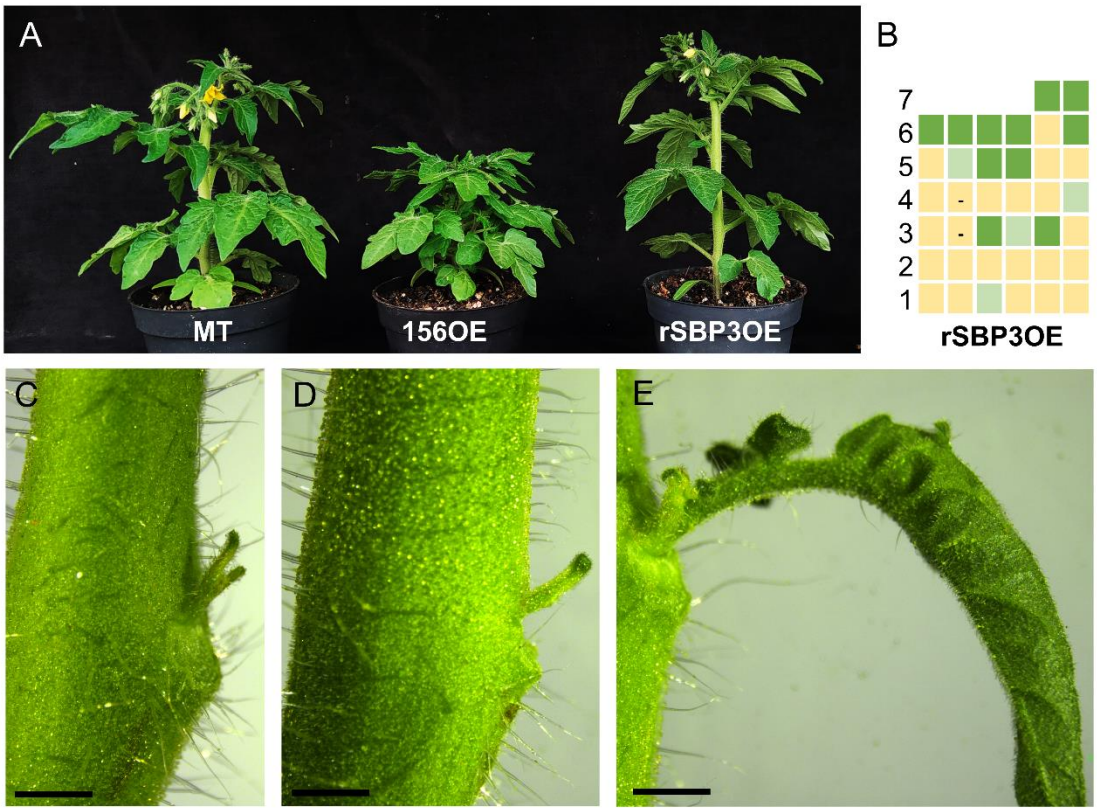

Figure S2. Phenotypes of 30-day old MT, 156OE and rSBP3OE plants (A). Branching pattern scheme of rSBP3OE plants (B). Axillary buds of MT (C) and rSBP3OE plants (D). In rSBP3OE plants, axillary buds sometimes form fully expanded leaves but do not elongate into a branch (E). Scale bars in C-E represent 2mm.

Figure S3. Representative phenotype of the hybrid F1 offspring resulting from the crossing between MT, 156OE and rSBP15OE plants with WT cv. Moneymaker plants at 39 (A) and 53 days after germination (B).

Table S1. qPCR primers used in this study

Primer	Sequence	Amplicon size	ID	Reference
SlPIN1-F	GCTGCAGGCTGGTCTAGATT	155	Solyc03g118740	Pattison and Catalá, 2012
SlPIN1-R	AACAATGGCAACAAAGCACA			
SlPIN4-F	AAAGAGGGACCCACTGGACT	121	Solyc05g008060	Pattison and Catalá, 2012
SlPIN4-R	TCATAACACTAGCCGGAGGC			
SlIPT3-F	TGTTCGTCATTTTCGCGGTG	90	Solyc01g080150	
SlIPT3-R	GTAGCAACTTTGTGCAAGAAGGA			
SlIPT4-F	AGTGAGATGCTGCTGCCATA	111	Solyc09g064910	
SlIPT4-R	TGGTGATGGGACAGAGCAGA			
LS-F	CATCGAGGCGTTGGATTATT	104	Solyc07g066250	
LS-R	TCCCAAACCACACTTGTTCA			
SlBRC1a-F	TGCTGATTCGGACAAACTAGA	155	Solyc03g119770	Martín-Trillo et al, 2011
SlBRC1a-F	GTCCTACACCAGCACTACCACT			
SlBRC1b-F	CTTCGAATTGCAACCACG	104	Solyc06g069240	Martín-Trillo et al, 2011
SlBRC1b-R	TTTTGAGATCCGTCCACTAATTC			
TUBULIN-F	TGAGGTCTTCTCACGCATTG	104	Solyc08g006890	
TUBULIN-R	CACTGAACTCGCCTTCTTCC			

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