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Characterization of Factors Regulating the End of Flowering in *Solanum lycopersicum*

María Jesús López Martín

Supervisors:
Cristina Ferrándiz Maestre
Mª Concepción Gómez Mena

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Department of Biotechnology

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Author: MARÍA JESÚS LÓPEZ MARTÍN

Supervisors
Dr. CRISTINA FERRÁNDIZ MAESTRE
Dr. MARÍA CONCEPCIÓN GÓMEZ MENA

Tutor UPV
MARÍA PILAR LÓPEZ GRESA

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ibmcp Instituto de Biología Molecular y Celular de Plantas

“A la voluntad, más que a la inteligencia, se enderezan nuestros consejos; porque tenemos la convicción de que aquélla, como afirma cuerdamente Payot, es tan educable como ésta, y creemos además que toda obra grande, en arte como en ciencia, es el resultado de una gran pasión puesta al servicio de una gran idea.”

Ramón y Cajal, «Reglas y consejos sobre investigación científica: Los tónicos de la voluntad.»

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Summary

Abstract

Flowering plants adjust their reproductive period to ensure reproductive success. This involves a tight control of flower initiation but also of the termination of the flowering period to optimize resource allocation for seed production. The end of flowering is marked by the cessation of the production of new flowers by the inflorescence meristems, that enter a dormant-like state known as proliferative arrest. This process has been mainly studied in Arabidopsis at the physiological, genetic and molecular levels, but remains to be characterized in other species to propose general mechanisms and provide the grounds to design biotechnological strategies aimed to control the duration of the fruit/seed productive season. *Solanum lycopersicum* (tomato) is an excellent model for this goal because of its economic importance but also the marked differences in plant architecture, meristem organization and fruit development. By comparing plants producing fertile and parthenocarpic seedless fruits, we have determined that proliferative arrest in tomato is a reversible process triggered by seed formation. We have identified the seeds as the likely source of signals that instruct the meristems to arrest in a coordinated and quantitative manner. The presence of auxin and abscisic acid in exudates from fertile but not from parthenocarpic fruits, and the effect on proliferative arrest of exogenous treatments with auxin on seedless fruits supports a major role of these phytohormones in the communication between seeds and meristems. On the other hand, we have studied the conservation of the FUL-AP2 pathway that controls the end of flowering in *Arabidopsis thaliana*. We developed molecular tools to modulate the expression of a *FUL* homolog in tomato (*MBP20*) and the sly-miR172, a negative regulator of AP2. By modulating these factors, we have obtained plants that show an early proliferative arrest in tomato. Our results indicate that the AP2-miR172 module is conserved between species, whereas the *MBP20* gene seems to have an opposite role, promoting meristem activity in this species. Our work supports the conservation of factors controlling proliferative arrest in flowering plants, while providing new insights into the regulation of the process.

Resumen

Las plantas con flores ajustan su periodo reproductivo para garantizar el éxito reproductivo. Esto implica un control estricto del inicio de la floración, pero también de la finalización del periodo de floración para optimizar la asignación de recursos para la producción de semillas. El final de la floración está marcado por el cese de la producción de nuevas flores por los meristemos de la inflorescencia, que entran en un estado de latencia conocido como detención proliferativa. Este proceso se ha estudiado principalmente en Arabidopsis a nivel fisiológico, genético y molecular, pero queda por caracterizar en otras especies para proponer mecanismos generales y sentar las bases para diseñar estrategias biotecnológicas destinadas a controlar la duración de la temporada productiva de frutos/semillas. *Solanum lycopersicum* (tomate) es un excelente modelo para este objetivo debido a su importancia económica pero también a las marcadas diferencias en la arquitectura de la planta, la organización de los meristemos y el desarrollo del fruto. Comparando plantas que producen frutos fértiles y partenocárpicos sin semillas, hemos determinado que la detención proliferativa en tomate es un proceso reversible desencadenado por la formación de semillas. Hemos identificado las semillas como la fuente probable de señales que instruyen a los meristemos a detenerse de forma coordinada y cuantitativa. La presencia de auxina y ácido abscísico en exudados de frutos fértiles pero no de frutos partenocárpicos, y el efecto sobre la detención proliferativa de tratamientos exógenos con auxina en frutos sin semillas apoya un papel importante de estas fitohormonas en la comunicación entre semillas y meristemos. Por otro lado, hemos estudiado la conservación de la ruta FUL-AP2 que controla el final de la floración, descrita en *Arabidopsis thaliana*. Hemos desarrollado herramientas moleculares para modular la expresión de un homólogo de *FUL* en tomate (*MBP20*) y el *sly-miR172*, un regulador negativo de *AP2*. Al modular estos factores, hemos obtenido plantas que muestran una parada proliferativa temprana en tomate. Nuestros resultados indican que el módulo AP2-miR172 está conservado entre especies, mientras que el gen *MBP20*, parece tener un papel opuesto, promoviendo la actividad del meristemo en esta especie. Nuestro trabajo respalda la conservación de los factores que controlan la parada proliferativa en plantas con flores, al tiempo que aporta nuevos conocimientos sobre la regulación del proceso.

Resum

Les plantes amb flors ajusten el seu període reproductiu per garantir l'èxit reproductiu. Açò implica un control estricte de l'inici de la floració, però també de la finalització del període de floració per optimitzar l'assignació de recursos per a la producció de llavors. El final de la floració està marcat per la cessació de la producció de noves flors pels meristems de la inflorescència, que entren en un estat de latència conegut com a detenció proliferativa. Aquest procés s'ha estudiat principalment en Arabidopsis a nivell fisiològic, genètic i molecular, però encara queda per caracteritzar en altres espècies per tal de proposar mecanismes generals i establir les bases per dissenyar estratègies biotecnològiques destinades a controlar la durada de la temporada productiva de fruits/llavors. *Solanum lycopersicum* (tomaca) és un model excel·lent per a aquest objectiu, degut a la seua importància econòmica, però també per les marcades diferències en l'arquitectura de la planta, l'organització dels meristems i el desenvolupament del fruit. Comparant plantes que produeixen fruits fèrtils i partenocàrpics sense llavors, hem determinat que la detenció proliferativa en la tomaca és un procés reversible desencadenat per la formació de llavors. Hem identificat les llavors com la font probable de senyals que instruïxen els meristems a detindre's de manera coordinada i quantitativa. La presència d'auxina i àcid abscísic en els exsudats de fruits fèrtils, però no en els fruits partenocàrpics, i l'efecte dels tractaments exògens amb auxina en fruits sense llavors recolzen un paper important d'aquestes fitohormones en la comunicació entre llavors i meristems. D'altra banda, hem estudiat la conservació de la via FUL-AP2 que controla el final de la floració, descrita en *Arabidopsis thaliana*. Hem desenvolupat ferramentes moleculars per a modular l'expressió d'un homòleg de FUL en tomaca (*MBP20*) i del *sly-miR172*, un regulador negatiu d'*AP2*. En modificar aquests factors, hem obtingut plantes que mostren una parada proliferativa primerenca en tomaca. Els nostres resultats demostren que el mòdul AP2-miR172 està conservat entre espècies, mentre que el gen *MBP20* sembla tindre un paper oposat, promovent l'activitat del meristem en aquesta espècie. El nostre treball dona suport a la conservació dels factors que controlen la parada proliferativa en plantes amb flors, al mateix temps que aporta nous coneixements sobre la regulació d'aquest procés.

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Reproductive Strategy of Monocarpic Plants

Living organisms have developed different reproductive strategies to ensure their survival and the continuity of their species. Plants can be divided, according to their reproductive strategies, into two broad categories: monocarpic and polycarpic. Polycarpic plants, such as perennials, go through multiple reproductive cycles during their lifetime, flowering and producing seeds several times. In contrast, monocarpic plants synchronize their life cycle around environmental cues and internal signals to maximize reproductive success during a single reproductive season, after which they go into senescence and die (Thomas, 2013)**.**

Monocarpic plants undergo an initial phase of vegetative growth before the transition to reproductive development. This step is tightly regulated by genetic and environmental factors to ensure that flowering occurs under optimal conditions (Cho et al., 2017; Huang et al., 2024; Maple et al., 2024). A second aspect of reproductive regulation, less understood but also important, is the regulation of the end of flowering, which involves the cessation of meristem activity and, thereby, the formation of new flowers and fruits, and thus it is also known with the term “proliferative arrest”. This process has been interpreted as a mechanism to optimize resource allocation to developing fruits and seeds, and also to determine the size of the progeny at the end of its life cycle (Balanzà et al., 2023; González-Suárez et al., 2020; Sadka et al., 2023)

The timing and duration of the reproductive phase has an impact on the number of fruits and seeds that the plant can produce. Thus, understanding the molecular and physiological mechanisms governing the end of flowering in monocarpic plants can provide new strategies for improving yield and crop resilience. Since most globally important crops, such as rice, wheat, tomato, and maize, are monocarpic, studying proliferative arrest in these plants is crucial for enhancing agricultural productivity.

Proliferative Arrest in *Arabidopsis thaliana*

Early studies in several species, including tomato and pea, showed that the end of the reproductive phase coincides with the cessation of meristem activity, and identify developing fruits and seeds as the source of signal triggering this arrest (Lockhart & Gottschall, 1961; Murneek, 1932). The concept of proliferative arrest in *Arabidopsis thaliana* was first explored in the 1990s, along with the related but distinct process of monocarpic senescence (Hensel et al. 1993; Hensel et al. 1994). In Arabidopsis, flower

production ceases first in the main inflorescence apex, followed shortly by all inflorescence branches, and the term 'Global Proliferative Arrest' (GPA) was defined to refer to this process (Hensel et al., 1994). The study of Arabidopsis mutants with reduced fertility showed a significant increase in the number of inflorescences and flowers in these plants, indicating that the shoot apical meristem (SAM) remained active for a longer period. In addition, it was reported that the pruning of fruits could reactivate meristematic activity in arrested meristems, reinforcing the connection between seed development and the regulation of the end of flowering (Hensel et al. 1994). A recent study revealed that the end of flowering takes place as an uncoordinated local arrest of inflorescences rather than globally coordinated arrest (Ware et al. 2020), and the term Proliferative Arrest (PA) was considered more appropriate to describe this process.

Although research on this topic in non-model species is very limited, recent studies have provided insight into the physiological and genetic mechanisms controlling proliferative arrest in *Pisum sativum,* revealing high conservation of the factors triggering arrest in Arabidopsis and pea (Burillo et al., 2024; Martínez-Fernández et al., 2024). In *Solanum lycopersicum*, early studies linked fruit and seed development to a cessation of plant growth (Murneek, 1926, 1932), but the physiological, genetic, and molecular factors that control arrest in this species remain unexplored.

Four main factors controlling PA have been identified so far: developing fruits, hormonal signals, plant age, and environmental signals (Balanzà et al., 2023; González-Suárez et al., 2020; Sadka et al., 2023).In the next sections, a brief overview of what is known about the contribution of these factors is provided (Fig I.1).

The Role of Fruits in Controlling Proliferative Arrest

Source organs, such as leaves, produce energy-rich compounds like sugars through photosynthesis and supply these resources to sink organs, which include meristems, developing fruits, and seeds(Aslani et al., 2020; Walker & Bennett, 2018). This distribution of resources between source and sink organs determines how energy is allocated within the plant, having an impact on the timing of reproductive arrest and the cessation of meristematic activity(Bangerth, 1989; Walker et al., 2021).

There are two main hypotheses regarding the influence of fruits/seeds on PA. The first hypothesis suggests that fruits act as a source of a mobile signal, described as a *"*death hormone*"*, that induces PA in the meristem (Noodén & Leopold, 1988; Proebsting et al., 1977). Therefore, developing fruits and/or seeds would produce signals that travel to the inflorescence meristems, inhibiting further floral initiation from these meristems. These signals would indicate that the plant has successfully initiated reproduction and should

reallocate its resources toward fruit growth and maturation rather than supporting further flower production. In support of this hypothesis, a recent study showed that hormonal signals from developing fruits, such as auxins, trigger the end of the reproductive phase by inducing meristem arrest (Walker et al., 2023; Ware et al., 2020), although it is still unclear how and at what level auxins work.

The second hypothesis proposes that fruits act as sink organs, redirecting the resources of the plant and indirectly inducing PA through nutrient competition (Bangerth, 1989). As fruits develop, they become strong sink organs, taking sugars, nutrients, and other key resources away from the meristems (Goetz et al., 2021). This resource limitation would result in the cessation of meristem activity, as the plant no longer has sufficient energy to support both fruit development and the initiation of new flowers (Davies & Gan, 2012; Kelly & Davies, 1988). In this model, the energy required for fruit development creates a significant demand on the plant's available resources, leading to meristem arrest as a resource-conserving mechanism.

While the "death hormone" hypothesis provides a direct regulatory mechanism, the source-sink dynamics offer a broader hypothesis to understand how the plant balances its energy needs during the critical phases of reproduction. There is evidence supporting both hypotheses, and neither has been conclusively ruled out; moreover, they may not be mutually exclusive. Determining the relative contribution of each mechanism (death hormone/nutrient imbalance) remains an open question, as they may work together to regulate the timing of reproductive arrest.

Hormonal Control of Proliferative Arrest

Hormones coordinate all the developmental processes of the plant, from vegetative growth to reproduction, and the basic components of the plant hormone action are conserved in the plant kingdom. This hormonal control is mediated by a complex interplay of several key hormones, including auxins (IAA), cytokinins (CKs), abscisic acid (ABA), and jasmonic acid (JA) (Cutler et al., 2010; Gasperini & Howe, 2024; Kieber & Schaller, 2018; Wasternack & Song, 2017). Increasing evidence supports the participation of phytohormones in orchestrating the process of proliferative arrest (PA) in monocarpic plants, regulating the transition from active meristem growth to reproductive termination (Fig I.1).

Auxins, in particular indole-3-acetic acid (IAA), are produced in developing fruits and seeds, and their role in regulating PA has been studied in recent years, particularly in *Arabidopsis*. One hypothesis suggests that once the inflorescence reaches a certain

state of competence, auxins produced by the proximal fruits are transported to the shoot apical meristem, where they induce PA. The precise mechanism of auxin action, although not fully understood, has been proposed to involve the disruption of auxin transport dynamics, potentially affecting auxin flow to the meristem and thus suppressing its activity (Ware et al., 2020). Although this work proposed that auxins produced by fruits induce inflorescence meristem arrest, later studies by the same authors raised doubts about this model. Walker et al. (2023) provided new evidence indicating that auxins may not directly arrest the inflorescence meristem activity but induce a developmental block of already initiated flower primordia. These observations, largely based on morphological data, are difficult to interpret and leave great uncertainty about how auxin signaling actually regulates meristem activity. Thus, while it is widely accepted that fruit-derived auxins trigger proliferative arrest, the molecular mechanism (via transport dynamics or another mechanism) remains unknown.

In contrast to auxins, cytokinins are generally associated with the promotion of cell division and meristem maintenance during early plant development (Gordon et al., 2009; W. Yang et al., 2021). During the vegetative phase, cytokinins support meristematic activity, driving growth and the formation of new leaves. After floral transition, cytokinin signaling is maintained in the inflorescence meristem, but as the plant progresses into the late stages of the reproductive phase, cytokinin levels in the meristem decline. This reduction in cytokinin signaling correlates with PA, as the decreased levels of cytokinins result in a low rate of cell division in the meristem, facilitating the transition to proliferative arrest (Merelo et al., 2022). Interestingly, exogenous cytokinin treatments of the inflorescence meristem prevent PA, and the meristems continue to produce new flowers and fruits. Moreover, in plants that have already entered PA, cytokinin application leads to the reactivation of meristematic activity (Balanzà et al., 2023; Merelo et al., 2022).

Transcriptomic studies of inflorescences in the context of PA have revealed that abscisic acid (ABA) also regulates meristem activity at the end of flowering (Martínez Fernández, 2017; Sánchez-Gerschon et al., 2024; Wuest et al., 2016). A sharp increase in ABA levels and ABA responses in the inflorescence apex correlates with the decline in CK signaling, suggesting that ABA promotes the transition to a dormant-like state as flowering ceases. Notably, several transcription factors involved in ABA signaling, such as *HOMEOBOX PROTEIN 21*, *40*, and *53* (*HB21*, *HB40*, and *HB53*), are up-regulated in the inflorescence apex at the end of the flowering phase (Sánchez-Gerschon et al., 2024). These factors promote ABA accumulation and response in the inflorescence apex and appear to affect specifically the developmental arrest of unpollinated flower buds, that form the typical floral cluster associated with PA (Balanzà et al., 2023; Sánchez-Gerschon et al., 2024).

ABA signaling has been mainly associated to the induction of dormancy in different developmental processes (Ali et al., 2022; Gonzalez-Grandio et al., 2017). For example, at the end of seed development, ABA accumulation inhibits cell division(Luo et al., 2023; L. Yang et al., 2014) and induces a dormant-like state of the embryo (Vishwakarma et al., 2017). A similar effect has been assigned to ABA in the control of axillary bud dormancy (Gonzalez-Grandio et al., 2017). Given the role of ABA in the induction of dormancy in axillary meristems, it is plausible that a similar mechanism takes place in the regulation of the apical meristem at the end of flowering. This would suggest that ABA could act as a key hormonal signal to trigger the cessation of inflorescence meristem activity, signaling the transition to a dormant state as part of the strategy of the plant to conclude its reproductive phase. Further studies are needed to explore in detail the molecular mechanism that explains how ABA triggers proliferative arrest.

In addition to CK and ABA, other hormonal signals may also influence the regulation of meristem arrest in Arabidopsis. Jasmonic acid (JA) is primarily recognized for its role in plant defense and stress responses, but it also plays a significant role in reproductive development (Dar et al., 2015; Wasternack & Song, 2017). During development, JA participates in the promotion of senescence and the cessation of floral activity, especially under stressful conditions (Wasternack & Hause, 2013). By inducing the early termination of meristematic activity when environmental conditions are suboptimal, JA ensures that the plant efficiently allocates its resources. This mechanism prevents unnecessary growth and helps the plant prioritize resource investment in fruit development when resources are limited (Dar et al., 2015). While this is suggestive of a putative role of JA in the control of proliferative arrest it has not been explored in detail and needs additional research.

Age-Genetic Pathway that Controls the End of Flowering in Model Species

In *Arabidopsis thaliana*, it has been described that the cessation of meristem activity at the end of flowering is controlled by an age-dependent genetic pathway (Fig I.1)**.** The first identified components of this pathway were the transcription factors *FRUITFULL* (*FUL*) and *APETALA2* (*AP2*) (Balanzà et al., 2018), which regulate *WUSCHEL* (*WUS*) expression in the meristem, a gene necessary for the maintenance of the stem cell niche (Mayer et al., 1998). *WUS* expression is maintained at the shoot apical meristem by AP2, while FUL directly and negatively regulates *AP2* and other AP2-like genes, all f them targets ofthe miR172 (Aukerman & Sakai, 2003; Zhu & Helliwell, 2011). As the plant ages, FUL and miR172 gradually accumulate in the inflorescence, leading to the

repression of *AP2-like* genes, which, in turn, causes the downregulation of *WUS*. The FUL-AP2 pathway regulates meristem activity at the end of the reproductive phase, preventing the formation of new flowers and allowing the plant to focus its resources on fruit development and seed maturation (Balanzà et al., 2018).

A connection between the *FUL-AP2* genetic module and the hormonal regulation of proliferative arrest has been identified. Transcriptomic analyses revealed that AP2 acts as a direct regulator of hormone signaling, controlling both abscisic acid (ABA) accumulation and signaling, and repressing cytokinin (CK) signaling. This establishes a clear link between AP2 activity and the modulation of these two hormones, which are regulators of meristem activity (Martínez-Fernández et al., 2020). Furthermore, Merelo et al. 2022 demonstrated that *FUL* also directly influences cytokinin levels at the SAM, reinforcing the idea that *FUL* and *AP2* work together to control cytokinin signaling during this developmental transition.

Role of Environmental Factors in Proliferative Arrest

Increasing evidence suggests that environmental factors are also involved in controlling the timing of proliferative arrest (PA) in monocarpic plants (Fig I.1), although this remains a relatively unexplored area compared to the more extensively studied genetic and hormonal regulation. The *APETALA2* (*AP2*) gene, a central element of the age-dependent pathway, interacts with environmental signals like far-red light and cold temperatures to regulate PA timing (Martínez-Fernández et al., 2020). Although much remains to be discovered, this ability of *AP2* to integrate environmental signals suggests that it plays a role in fine-tuning the arrest of meristematic activity in response to changing conditions.

The *FLOWERING LOCUS T (FT)* gene is one of the most important factors that integrate environmental signals such as photoperiod and temperature in the control of flowering time (Andrés & Coupland, 2012; Kobayashi et al., 1999) but interestingly, it has also been shown to contribute to the regulation of the end of the reproductive phase. *FT* expression increases throughout flowering, reaching a peak at inflorescence meristem arrest. In *Arabidopsis thaliana*, *ft* mutants extend the duration of the flowering phase and increase the number of flowers produced by the inflorescence, indicating that *FT* acts as a negative regulator of meristematic activity (González-Suárez et al., 2023).

Understanding how environmental factors influence the regulation of the lifespan of monocarpic plants is of great interest. However, much remains to be explored about how these processes are integrated, making this an important area for future research.

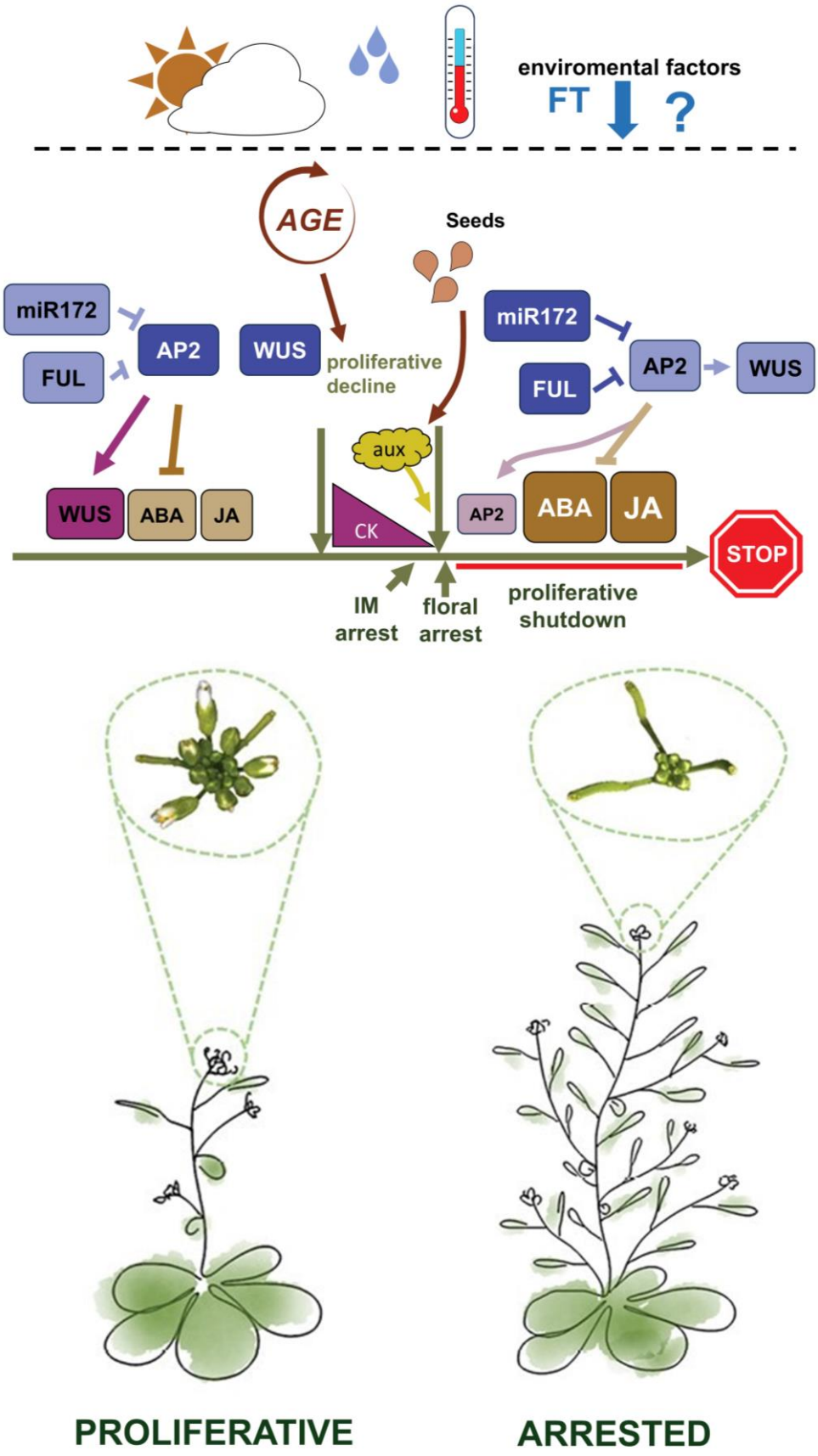


Figure I.1. Regulatory factors and morphological changes associated with proliferative arrest in Arabidopsis. Environmental factors influence the timing of both the initiation and

termination of flowering, with partial involvement of FT. Internal signals, particularly plant age and seed production, play critical roles in determining the cessation of meristem activity. During the early stages of the reproductive phase, cytokinin (CK) signaling, along with meristem-maintaining transcription factors such as AP2 and WUS promote a proliferative state in the inflorescence meristem. Conversely, age-related factors like miR172 and FUL, which act as negative regulators of AP2, are present at low levels during this phase. As the plant transitions to the late flowering stage, marking the onset of proliferative decline (as described by Merelo et al., 2022), the accumulation of miR172 and FUL leads to a reduction in CK, *AP2*, and *WUS* levels. The suppression of *WUS* expression corresponds with a decline in meristem activity, ultimately leading to the proliferative arrest, where *WUS* is undetectable. This reduction in *AP2* also triggers increased abscisic acid (ABA) and jasmonic acid (JA) signaling, driving the meristem into a dormant-like state. Additionally, auxin exported from mature fruits promotes floral arrest by suppressing meristem activity (Walker et al., 2023). Morphologically, the inflorescence meristem shows distinctive changes between the proliferative and arrested states. In the proliferative phase, the apex actively produces floral organs, showing a wide range of developing buds. By contrast, in the arrested state, the cessation of further floral development results in a cluster of unopened flower buds. Adapted from Balanzá et al., 2023.

Relevance of Tomato Cultivation

Tomato (*Solanum lycopersicum*) is one of the most cultivated and economically significant vegetable crops globally. According to recent data from the FAO, global tomato production exceeds 180 million tons annually, making it one of the most widely grown and consumed vegetables. This crop is fundamental to the agricultural economies of many regions, and in Europe Italy and Spain are the main producers. Spain produced approximately 4 million tons of tomatoes in 2022, making it one of Europe’s leading tomato exporters (Fig I.2).

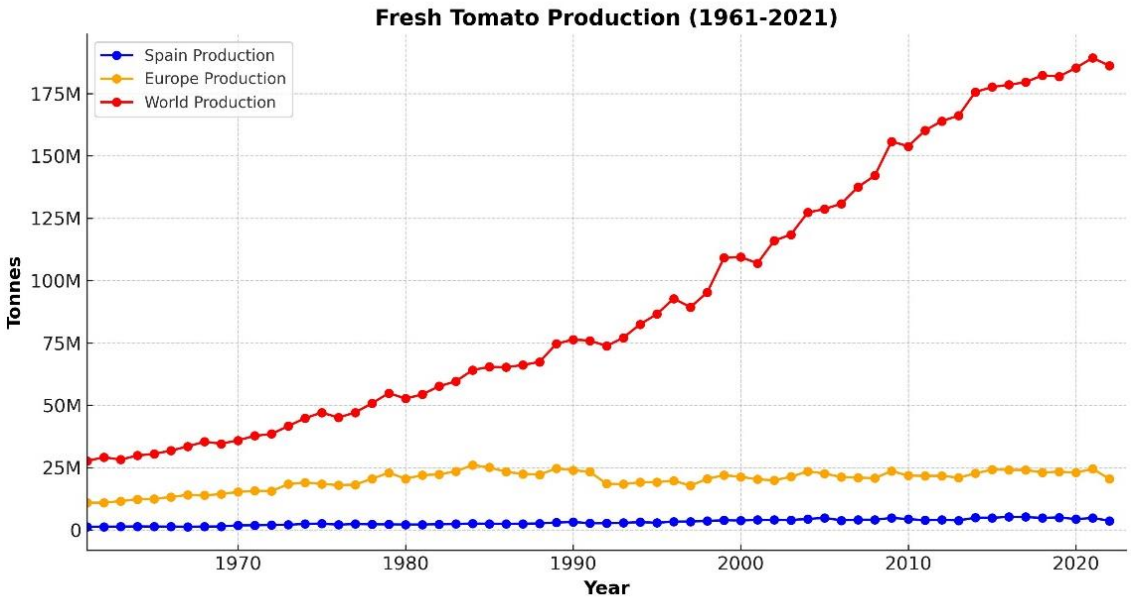


Figure I.2. Production of fresh tomato (in tons) from 1961 to 2021 in Spain, Europe, and the world. M indicates millions of tons. Graph modified from data obtained from FAOSTAT (October 2024) at https://www.fao.org/faostat/en/#compar.

Tomato is cultivated in a wide range of climates and geographical regions, from large commercial operations in temperate zones to smaller-scale farms in subtropical areas. This diversity in cultivation is reflected in the vast array of cultivars that have been developed over the years. There are hundreds of tomato cultivars available worldwide; each specifically selected for different traits, including variations in fruit size, shape, color, flavor, and growth habits (Foolad, 2007; Peralta & Spooner David M., 2006). These cultivars can be broadly categorized into traditional varieties, which are often valued for their distinctive flavors and diverse appearances, and commercial hybrids, which have been designed for characteristics such as disease resistance, uniformity, and higher yields (Bai & Lindhout, 2007a; Naika, 2005; Tieman et al., 2006).

Beyond the economic relevance, tomatoes are important for human nutrition as a source of vitamins and other nutrients due to their high content of vitamins A and C, antioxidants like lycopene, and other essential nutrients (Raiola et al., 2014). Lycopene, in particular, has been extensively studied for its potential health benefits, including its antioxidant properties and possible protective effects against cardiovascular diseases and certain cancers (Chen et al., 2015; Eliassen et al., 2012; Holzapfel et al., 2013; Raiola et al., 2014). This has further increased consumer demand for tomatoes, driving innovation in both cultivation practices and grower breeding programs.

Advances in genetic modification, agriculture, and biotechnology will contribute to optimizing tomato production, ensuring that this essential crop can meet the demands of consumers in a changing climate.

Tomato Architecture: Determinate and Indeterminate Tomato Cultivars.

The cultivated tomato is a monocarpic plant that exhibits a sympodial growth pattern in which the growth of the main stem is maintained by the development of sympodial structures that are reiterated over time. The vegetative phase of growth begins with the development of the primary shoot meristem (PSM) generating approximately 7 to 12 leaves before switching to the reproductive phase. At this stage, the PSM develops into a transitional meristem (TM), which rapidly transforms into a floral meristem (FM) and inflorescence meristem (IM) (Figure I3.A). A determinate compound inflorescence develops from these meristems, which follows a zigzag pattern (Lippman et al., 2008).

After the growth of the PSM is terminated, new lateral shoots, known as sympodial meristems (SYM), emerge from the axil of the last leaf. Each SYM produces 1 to 3 leaves, develops an inflorescence and finally terminates. A new SYM is then activated in the axil of the last leaf of the previous sympodial unit, ensuring the continuation of plant growth. This pattern is repeated over time, defining the sympodial growth architecture of the tomato plant (Park et al., 2012).

According to their growth habit, tomato cultivars (Figure I.3B) are classified into determinate, indeterminate, and semi-determinate (Fridman et al., 2002). In indeterminate cultivars, the sympodial meristems continue to produce new shoots indefinitely, allowing the plant to continue flowering and producing fruit for longer periods. These cultivars are particularly valued in greenhouse production and fresh produce markets because of their continuous production and their higher yield potential. In contrast, determinate cultivars carry loss-of-function mutations in the *SELF-PRUNING* (*SP*) gene, which causes the sympodial meristem to become a terminal inflorescence after a few sympodial units, thereby ceasing the development of new sympodial meristems and leading to plant growth determination (Pnueli et al., 1998; Thouet et al., 2008). This results in a more compact plant architecture suitable for mechanized harvesting, as growth ceases after the formation of a certain number of sympodial units. In these varieties, further growth and flowering can only continue from axillary meristems at the base of the plant.

Semi-determinate cultivars represent an intermediate growth pattern. These plants terminate after producing about eight inflorescences, achieving a balance between continuous fruiting and a more manageable plant size. Semi-determinate varieties maintain fruit production longer than determinate varieties, but their growth is not as extensive as indeterminate varieties. This growth habit allows moderate yields with less intensive pruning and support, making them a practical choice for growers looking for a compromise between productivity and ease of management (Vicente et al., 2015). Interestingly, recent studies have shown that certain *sp* alleles, such as the newly identified *sp-5732*, can delay shoot termination and extend leaf production, mimicking the heterosis-like effect seen in heterozygous *sft* mutants. This subtle suppression of determinacy, achieved by adjusting the florigen–anti florigen balance, can enhance yields by up to 42% compared to traditional determinate varieties, presenting a promising strategy for developing high-yield, semi-determinate field cultivars (Kang et al., 2022).

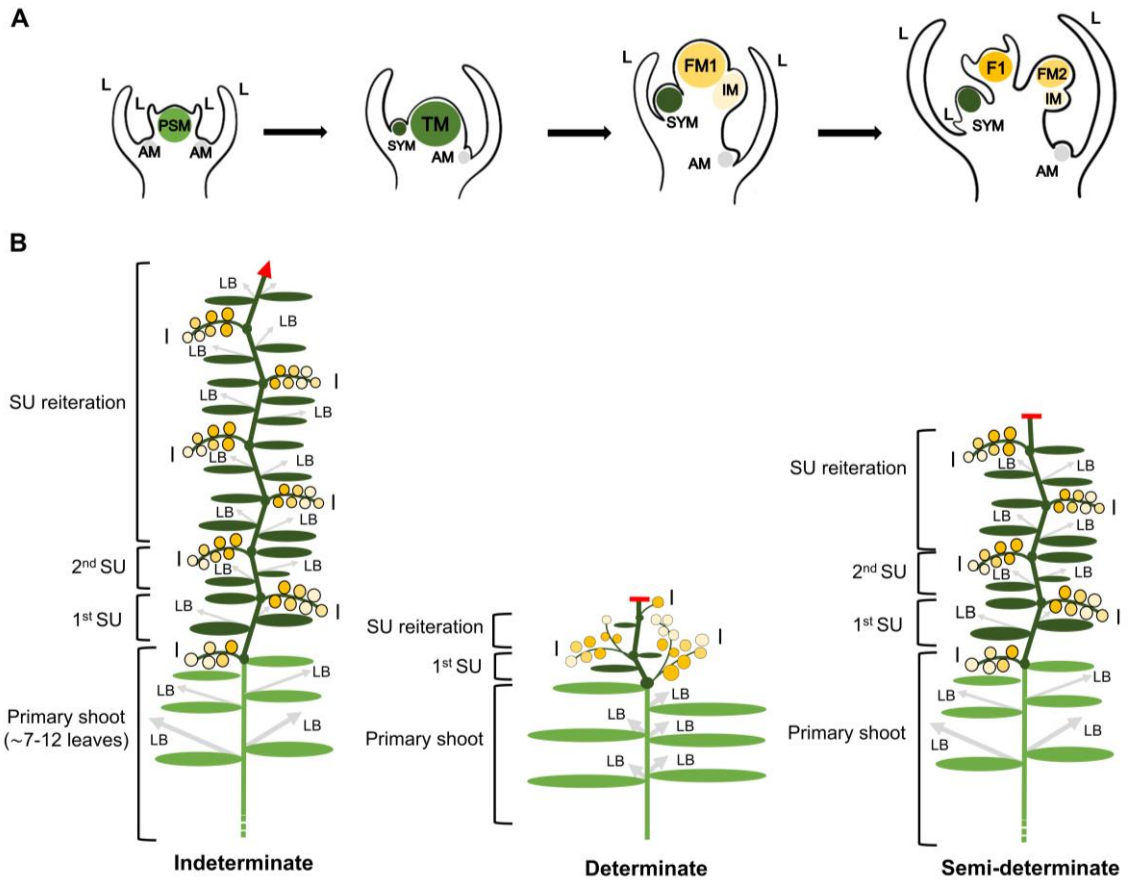


Figure I.3. Tomato sympodial growth and inflorescence development. A) Primary shoot meristem (PSM) maturation and development. During the vegetative stage, the PSM produces vegetative phytomers (leaves and axillary meristems, AM). Upon transitioning to flowering, the PSM becomes a transitional meristem (TM) that develops into the first floral meristem (FM1) and the inflorescence meristem (IM1). This process continues in a repetitive manner, resulting in a

compound inflorescence with a zigzag pattern of flower initiation. Simultaneously, a specialized meristem called the sympodial meristem (SYM) forms in the axil of the last leaf, leading to the first sympodial unit, which reiterates over time. B) Three distinct growth habits of tomato plants are represented: indeterminate, determinate, and semi-determinate. Indeterminate plants show continuous growth, as indicated by the ongoing production of sympodial units along the main shoot. Determinate plants, in contrast, cease growth after a set number of inflorescences, with sympodial units terminating at the shoot apex. Semi-determinate plants display a growth pattern intermediate between indeterminate and determinate, terminating growth after a reduced number of sympodial units and inflorescences. Leaves are represented by diagonal lines and inflorescences by clusters of circles along the main stem.

A prominent model of the determined cultivar is Micro-Tom, a miniature variety widely used as a model system for the study of tomato fruit set. Micro-Tom was developed for ornamental purposes and is characterized by its dwarf stature due to mutations in the *SELF-PRUNING* (*SP*) and *DWARF* (*D*) genes. The mutation in the *SP* gene results in a determinate growth phenotype, while the mutation in the *D* gene reduces internode length and leads to smaller, wrinkled, dark-green leaves due to a decrease in brassinosteroid (BR) content. Additionally, Micro-Tom may have a third, uncharacterized mutation (*miniature*, *mnt*; Meissneret al., 1997) that does not affect gibberellin (GA) metabolism but is likely linked to GA signaling. Micro-Tom is very valuable for molecular genetics research, especially because it is highly susceptible to genetic transformation and because it can be grown in confined spaces, making it ideal for high throughput studies (Martí et al., 2006). In addition, in this genetic background, numerous collections of mutants, transgenic lines and CRISPR-edited variants have been developed. These resources are widely used to study key aspects of plant development, such as the regulation of flowering, fruit set, and hormonal control of growth (Barrera-Rojas et al., 2023; Ezquerro et al., 2023; Lee et al., 2024; Serrani et al., 2010).

Biotechnological Strategies for Enhancing Crop Productivity and Sustainability

Understanding the mechanisms regulating proliferative arrest (PA) in monocarpic plants, such as tomato, would be useful for developing innovative biotechnological strategies to improve crop productivity. By manipulating PA, it may be possible to prolong the reproductive phase, increase yields, or optimize harvest cycles in economically important crops. In species such as pea, studies have already demonstrated the potential of altering PA to influence flowering and fruiting times (Martínez-Fernández et al., 2024), providing valuable insights into how these mechanisms could be applied to other crops, including tomato.

Tomato is a valuable model organism for studying PA, not only because of its importance as a crop, but also because it belongs to the Solanaceae family, which includes many horticulturally important species. The knowledge gained from understanding how PA is regulated in tomato could have broader applications in solanaceous crops such as pepper, eggplant, and potato, which represent 39% of horticultural production in the world (Morris and Taylor 2017). In this context, this thesis contributes to the growing field of research on proliferative arrest, offering insights that could inform future biotechnological approaches to improve crop yield and sustainability.

Objectives

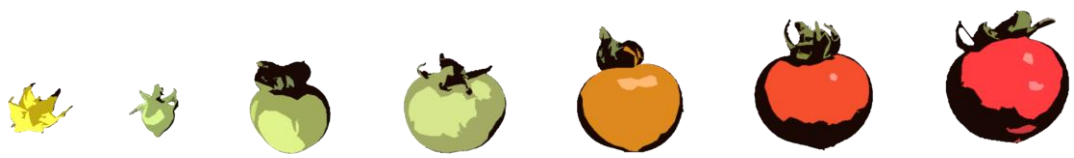
Objectives

The global objective of this thesis is to characterize the end of flowering at the physiological, genetic, and molecular levels in *Solanum lycopersicum*, identifying the mechanisms that control the arrest of tomato shoot meristem activity and the production of new flowers, and how it is controlled by fruit set and seed formation.

To this purpose, we propose the following specific objectives:

1. Characterize proliferative arrest in tomato plants at the physiological level in indeterminate and determinate cultivars.
2. Identify and define the source(s) and nature of the signal(s) controlling meristem arrest dependent on fruit and seed formation in tomato.
3. Characterize meristem arrest in tomato at the genetic level, by assessing the conservation of the age-dependent FUL-AP2 pathway, first described in *Arabidopsis*.

Chapter 1



[1] This Chapter has been submitted and is currently under review at Plant Physiology with the reference: López-Martín, M. J., Ferrándiz, C., & Gómez-Mena, C. (under review). The end of flowering in tomato is triggered by the quantitative effect of seed production. *Plant Physiology*.

The end of flowering in tomato is triggered by the quantitative effect of seed production

Introduction

Flowering plants adjust their reproductive strategies to optimize the success of their offspring. This involves the coordination of reproductive development with environmental conditions such as light, water, temperature, and the availability of pollinators. As a result, flowering initiation is tightly controlled by these factors, as well as by endogenous signals that ensure that the plant is able to support reproduction. In addition, the end of the flowering period is also a regulated process, a fundamental evolutionary adaptation that ensures nutrient availability and redistribution for seed production while optimizing the size of the progeny (Balanzà et al., 2023).

In monocarpic plants, those with a single reproductive cycle, the end of the flowering period marks the cessation of the activity of inflorescence meristems: no more floral primordia are produced, no new flowers open, and the plant completes fruit filling before initiating a global senescence program that culminates in organismal death. This process, known as proliferative arrest (PA), has been described in many species of agronomic interest including tomatoes, grasses or legumes (Biswas & Choudhuri, 1980; Lockhart & Gottschall, 1961; Murneek, 1926). These early works proposed that the development of fruits and seeds are the major factor triggering arrest, but did not clarify the mechanisms involved. Some authors suggested that fruit and seed development could be acting as strong sinks causing a nutritional imbalance in the meristem, while others suggested the existence of a transmissible signal moving from seeds, a death hormone, that would instruct the meristems to arrest (Kelly & Davies, 1988; Wilson, 1997). Despite its ecological and economic importance, the regulation of flowering cessation has been an overlooked topic, and only recently a number of independent studies have advanced our knowledge of PA regulation beyond these early works, providing the first molecular and genetic studies on the process (Balanzà et al., 2023; González-Suárez et al., 2020)

Most of the recent studies on PA regulation have been carried out in Arabidopsis. A seminal work by Hensel et al., 1994determined that Arabidopsis mutants with highly reduced fertility or plants where fruit formation was prevented showed a delay in PA. Moreover, the arrested inflorescence meristems could be reactivated by fruit removal,

supporting the hypothesis of a long-distance repressive signal from fruit/seeds that triggers PA (Hensel et al., 1994). Later it was shown that the fruit/seed effect appears to be active only when inflorescences have acquired the competence to arrest, suggesting that the mechanism that mediates this effect could be dependent on the age of the inflorescence. It has also been reported that PA can be separated into two phases: first, the inflorescence meristems (IM) cease to form new floral primordia (IM arrest), and second, the development of already formed but unpollinated floral buds is blocked (floral arrest) (Sánchez-Gerschon et al., 2024; Walker et al., 2023). Auxin exported from fruits was proposed to trigger PA in Arabidopsis and was therefore considered a candidate death hormone (Ware et al., 2020), but later it was found that its role is restricted to influencing floral arrest (Walker et al., 2023).

The recent advances in the physiology and genetic control of PA remain to be extended to other monocarpic species, especially crops that could benefit from this knowledge to design biotechnological strategies aimed to extend the productive cycle and thus yield. In this context, *Solanum lycopersicum* (tomato) holds great interest because of its economic importance, but also the significant differences with respect to Arabidopsis in plant architecture, meristem organization and fruit development. Unlike Arabidopsis, a plant with monopodial architecture, the development of the tomato plant is determined by the compound sympodial shoot growth. Arabidopsis shoot apical meristem produces leaves until the floral transition, when it becomes an inflorescence meristem (IM) that forms floral meristems on its flanks (FMs) resulting in a simple indeterminate inflorescence. However, in tomato plants, the compound inflorescence is determinate showing a zigzag pattern of flower formation (Lippman et al., 2008), and the development of new leaves and inflorescences is given by the sympodial shoot (SYM).

The primary shoot meristem (PSM) in tomato produces between 7 and 12 leaves before switching to reproductive growth and developing into a transitional meristem (TM). This ephemeral meristem quickly develops into a floral (FM) and an inflorescence meristem (IM). Successive iteration of this process gives the determinate compound inflorescence of tomato. Then, growth in the main axis continues from the formation, in the axil of the last leaf, of a specialized meristem called sympodial shoot meristem (SYM). This meristem develops about three vegetative nodes and determines into a new FM and IM. This pattern is repeated through time, giving rise to the tomato sympodial shoot (Supplementary Fig. 1.S1). Tomato plants have diversified into hundreds of cultivars that could be classified into two main growth habits, determinate and indeterminate. In indeterminate growth cultivars, the development of new sympodial units is repeated

indefinitely, while in determinate growth cultivars the mutation in the *SELF-PRUNING* (*SP*) gene results in the premature conversion of the SYM into a terminal determinate inflorescence. In this last case, growth and the formation of new inflorescences continues from axillary meristems of basal leaves (Park et al., 2012; Pnueli et al., 1998; Szymkowiak & Irish, 2006).

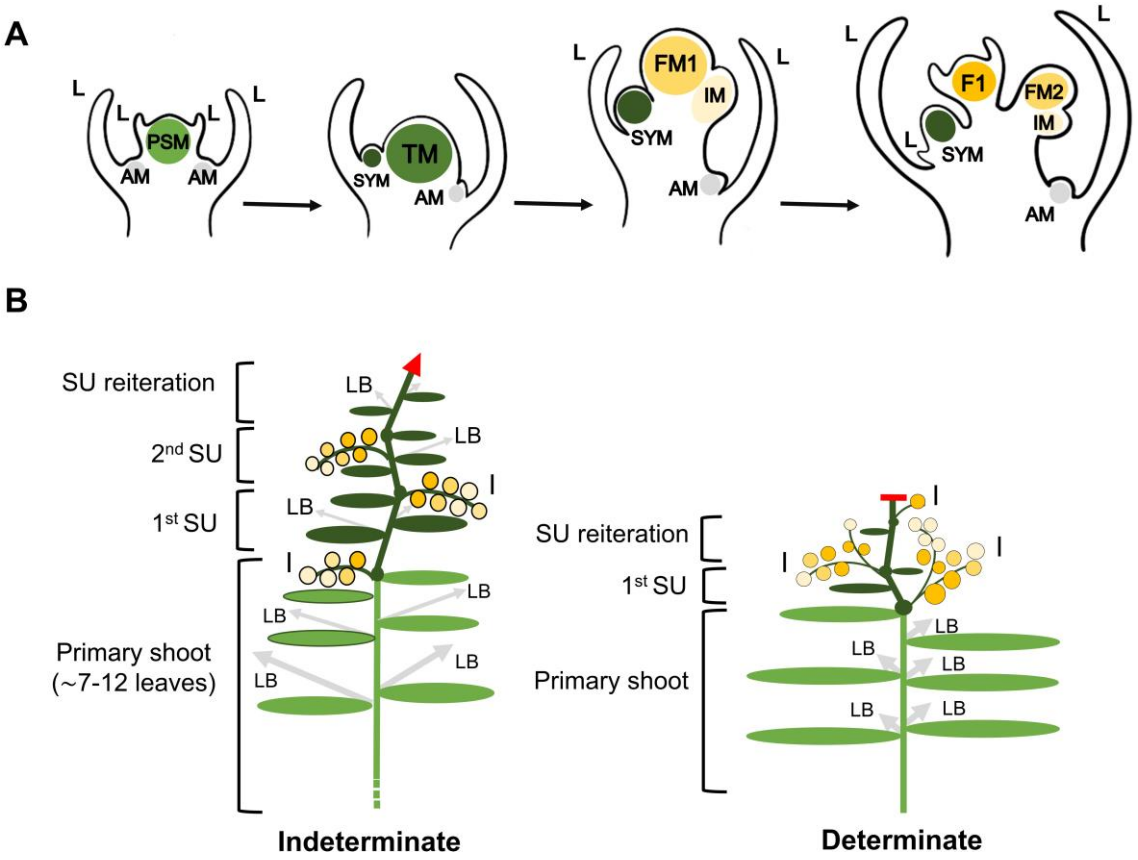


Figure 1.S1. Tomato sympodial growth and compound inflorescence development. A) Primary shoot meristem (PSM) maturation and development. During the vegetative stage, the PSM develops vegetative phytomers of leaves and axillary meristem (AM). At the transition to flowering the PSM becomes a transitional meristem (TM) that quickly develops into the first floral (FM1) and the inflorescence meristem (IM1). Successive iteration of this process gives the compound inflorescence of tomato showing a zigzag pattern of flower initiation. In parallel, in the axil of the last leaf, a specialized meristem called sympodial meristem (SYM) is formed, which will give rise to the development of the first sympodial unit. The sympodial units reiterate in time, following the same pattern. B) The two common growth habits in tomato plants are represented in two diagrams: indeterminate and determinate. Leaves are shown in green, lateral branches (LB) in grey and inflorescences (I) in yellow. After the development of the primary shoot, sympodial units develop. This structure is repeated along plant architecture. The continuation of growth in cultivars of indeterminate growth habit is indicated by a red arrow, and the cessation of growth in cultivars of determinate growth habit is shown by a red line at the upper part of the plant. Color patterns are used to relate the meristems in panel A to the organs that are formed from them in panel B.

Tomato plants produce fleshy fruits that grow and mature transitioning from partially photosynthetic to true heterotrophic tissues through the parallel differentiation of chloroplasts into chromoplasts and thus acting as strong sinks to accumulate sugars and other organic compounds in their ripe stage (Bangerth, 1989; Durán-Soria et al., 2020). Tomato was one of the first species where the influence of fruits and seeds was connected to reproductive development and the end of flowering. Already in the early 20th century, fruit development was associated with a decrease in sympodial meristem development (Murneek, 1926), while the initiation of embryo development was correlated with the decline in growth rate and senescence of the vegetative parts of the plant (Murneek, 1932). However, these studies were largely discontinued and failed to clarify the relative contribution of fruits and seeds to the control of proliferative arrest and the activity of the sympodial units.

In this work we have characterized the influence of fruit and seed development in the control of the end of flowering in tomato. Importantly, we have generated a mutant that produces seedless parthenocarpic fruits. These mutants have allowed us to study PA in the presence of ripening fruits without seeds, assessing the importance of fruits and seeds as putative sinks or sources of fruit-derived signals that could be triggering PA in this crop. Our results provide solid evidence for a major role of developing seeds in triggering PA and point to auxins as a key signal in this process.

Results

Role of fruits and seeds in the control of the end of flowering in tomato plants

Classical studies in tomato plants identify fruit development as an important factor promoting the cessation of the proliferative capacity of meristems, which in turn puts an end to flower and fruit production (Murneek, 1926). To better understand the quantitative effect of fruit development on the control of this process, we conducted flower pruning experiments that prevent fruit set. We compared wild-type plants in which different treatments of flower pruning were implemented: untreated plants, plants where every other flower was removed (1/2), and plants where all flowers were removed (Fig. 1.1A).

Untreated wild-type plants reached proliferative arrest 7 weeks after the floral transition, while plants in which the formation of half of the fruits was prevented remained active for a further five weeks, arresting after 12 weeks. Interestingly, the set of plants in which fruit formation was completely avoided recurrently activated new axillary meristems that gave rise to new flowers and consequently did not undergo PA (Fig. 1.1B-C). Pruning experiments indicated that, in tomato plants, the timing of PA is a quantitative character controlled by the presence of developing flowers and fruits. Accordingly, and regardless of the duration of the reproductive period, both groups of arrested plants produced a very similar number of fruits (~20) and seeds (~300) once PA was established (Fig. 1.2B-C), suggesting that a critical number of fruits and seeds triggers axillary meristem arrest in tomato.

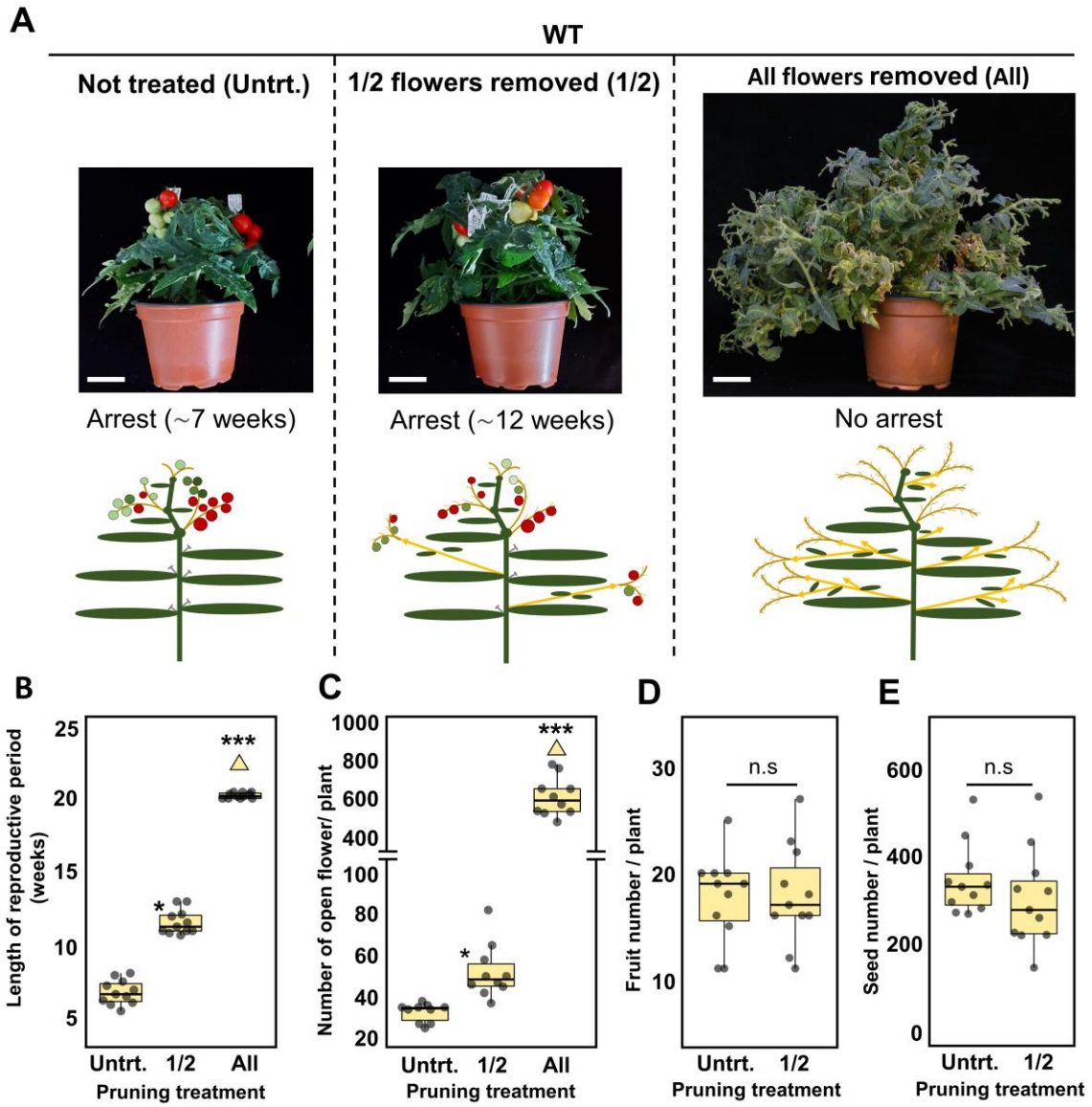


Figure 1 . 1. Effect of gradual flower pruning on proliferative arrest. A) Plant phenotype at the end of pruning treatments: untreated plants (Untrt.), plants where every other flower was removed (1/2), and plants where all flowers were removed (All). Effect of the treatments on **B)** the length of the reproductive period measured in weeks, **C)** the number of total open flowers per plant, **D)** the number of fruits per plant, and **E)** the number of seeds per plant. The yellow arrow in graphs B and C indicates that fully pruned plants (All) remain proliferative indefinitely and the end of flowering did not occur. Asterisks indicate significant differences according to *Kruskal-Wallis* test. **P < 0.05; ***P < 0.001*. Data correspond to n=11 independent plants. Scale bars = 5 cm

In agreement with the role of the fruits as a putative source of signals triggering PA, we found that axillary meristems of arrested wild-type plants were reactivated after all fruits were removed from the plant. These plants produced new flowers and fruits from lateral branches and arrested again once the critical number of fruits (~20) and seeds (~300) equivalent to the first meristematic arrest was reached (Fig 1.2A-C). This result reinforced the idea that fruits and seeds are potential sources of signals that trigger the end of flowering in tomato plants.

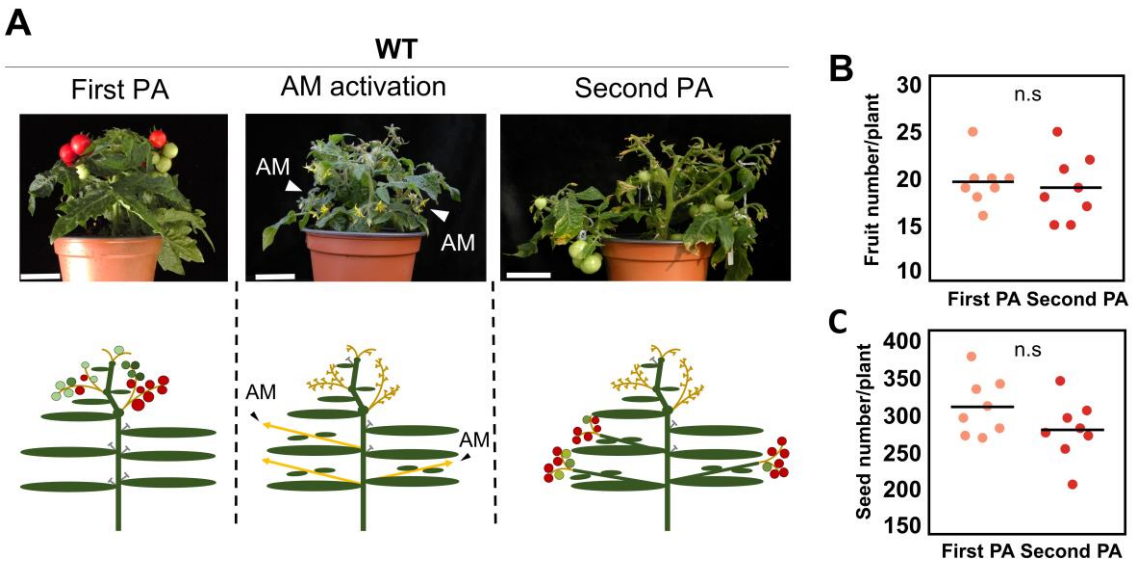


Figure 1.2. Fruits keep axillary meristems of tomato plants arrested. A) Fruit removal from arrested plants (First PA) causes the activation of the arrested axillary meristem (AM). B) Fruit and C) seed number per plant at PA before and after fruit removal. No significant differences were inferred according to Student's t-test ($P < 0.05$). Data correspond to n=8 independent plants. Scale bars = 5 cm

To assess the relative importance of fruits and seeds in the control of PA, we generated a tomato mutant bearing seedless (parthenocarpic) fruits. We previously showed that male sterility results in the formation of seedless fruits in different tomato cultivars (Medina et al., 2013; Rojas-Gracia et al., 2017; Salazar-Sarasua et al., 2022). Among the genes involved in pollen development, *ABORTED MICROSPORES* (*AMS*) encodes a basic helix-loop-helix (bHLH) transcription factor essential for pollen formation and maturation in Arabidopsis and tomato (Bao et al., 2022; Sorensen et al., 2003). Therefore, the *Solyc08g062780* (*SlAMS*) gene was selected as a target to generate male-sterile and parthenocarpic tomato plants using CRISPR/Cas9. We obtained a *SlAMS* knockout mutant in the Micro-Tom cultivar containing a single nucleotide insertion (+A) in the coding sequence of the gene (fifth exon) (Fig. 1.3A).

We compared the development of anthers of the wild type and the *Slams* mutant at four different floral stages: stage 8 (0.2cm), stage 10 (0.3cm), stage 14 (0.4cm), and stage 16 (0.6cm) according to (Brukhin et al., 2003) (Fig. 1.3 B-I). At stage 8, the epidermis, endothecium, middle cell layers, sporogenous cells, and tapetum were formed in both genotypes. In the mutant, we observed that sporogenous cells seemed slightly rounded and disorganized compared to the wild type (Fig. 1.3B). At stage 10, in wild-type anthers, meiosis was completed, resulting in tetrads. In the *Slams* mutant, sporogenous cells enlarge and continue to divide occupying the entire locule cavity (Fig. 1.3G). In the successive stages (stages 14 and 16), microspores completed their development into mature pollen grains in wild-type anthers (Fig. 1.3D-E). From stage 14, in the *Slams* mutant we observed severe degeneration of sporogenous cells and vacuolated tapetum (Fig 1.3H). Mature anthers of the *Slams* mutant showed the deposition of cell debris without viable pollen, resulting in complete male sterility (Fig. 1.3I).

Slams mutant developed seedless parthenocarpic fruits with no difference in fruit ripening time (Fig. 1.3J), although the weight of ripe fruits was reduced by 50% compared to the wild type (Fig. 1.3K). Total sugar levels were estimated by measuring the soluble solids content (ºBrix) of wild-type and parthenocarpic ripe fruits, which indicated a ºBrix index two points higher in seedless fruit (Fig. 1.3L). Since the *Slams* mutant showed complete parthenocarpy and seeds were never obtained, it was an ideal genotype to elucidate the role of seeds at the end of flowering in tomato.

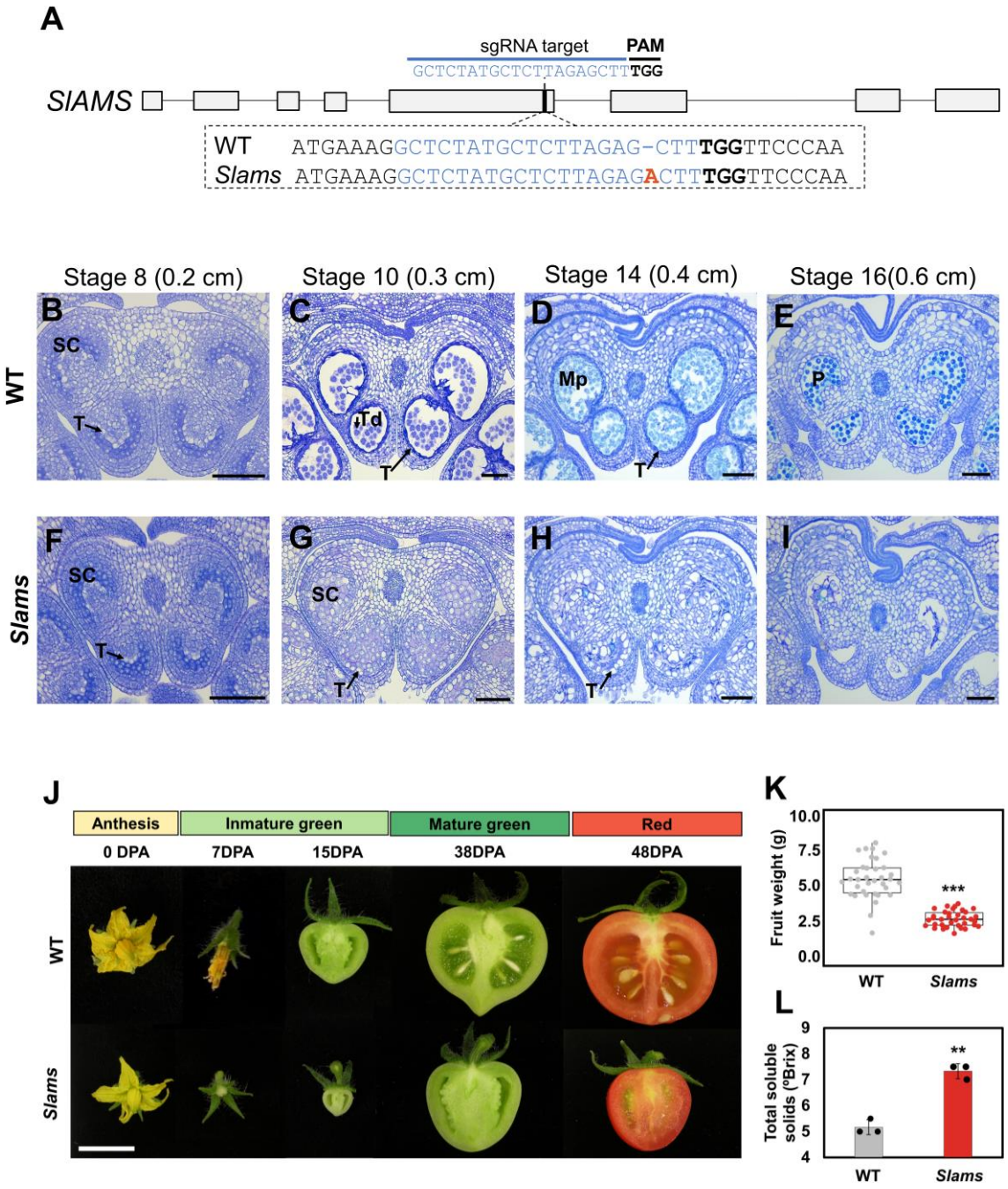


Figure 1.3. Knockout of *SlAMS* causes male sterility and parthenocarpy. A) A single guide RNA (in blue) was targeted to the fifth exon of the *Solyc08g062780* (*SlAMS*) gene. *Slams* mutant sequence (+A) is highlighted on the box (in red) **B-I)** Histological cross sections of anthers from the wild type (WT) and *Slams* mutant at different developmental stages (stage 8, 10, 14, and 16) named according to Brukhin et al., 2003. Scale bar: 100 µm. Abbreviations: Mp, microspores; P, mature pollen; SC, sporogenous cells; T, tapetum; Td, tetrads. **J)** Fruit development of the wild type and *Slams* mutant from anthesis (0 DPA) to ripe stage (48 DPA) DPA= days post anthesis. Scale bar: 1 cm. **K)** Ripe fruit weight of wild-type and *Slams* plants. The mean weight of n=35 fruits/ genotype is plotted. **L)** Total soluble solids content (°Brix) of ripe fruits of the wild type and *Slams* mutants. The mean value ± standard deviation of three biological replicates per genotype

(n=15 fruits/replicate) is shown. Asterisks indicate statistically significant differences according to Student's *t-test (**P<0.01, ***P<0.001*).

We monitored the production of flowers and fruits in the mutant compared to the wild type and to tomato plants where all flowers were removed to prevent fruit set (Fig. 1.4A). Wild-type plants showed a decreased flower production rate from week 4 after the floral transition until week 7 when proliferative arrest took place. The parthenocarpic mutant (*Slams*) that produces fruits but no seeds, did not undergo proliferative arrest. This continuous flower production was maintained by the initiation of new inflorescences, and the number of flowers per inflorescence was unaffected (Supplementary Fig. 1.S2).

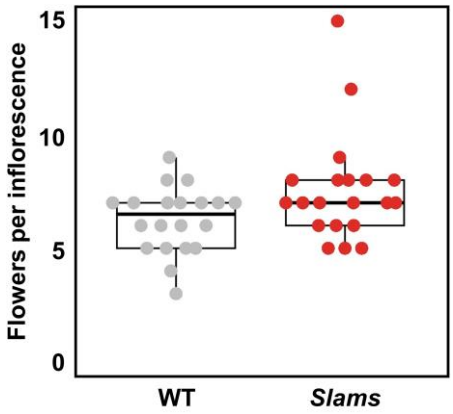


Figure 1.S2. **Number of flowers per inflorescence of *Slams* and wild type (WT).** Data are shown for n=20 inflorescences per genotype. No significant differences were inferred according to the Mann-Whitney U test.

Slams mutants and wild-type plants in which all flowers were removed to prevent fruit set remained proliferative over time and activated lateral branches with new inflorescences and flowers indefinitely (Fig. 4B). Consequently, seedless mutants produced fruits continuously. At the time the experiment was terminated, *slams* mutants had produced an average of 45 fruits, more than double of wild-type production, and plants were still activating new inflorescences, with no sign of proliferative arrest (Fig. 4C).

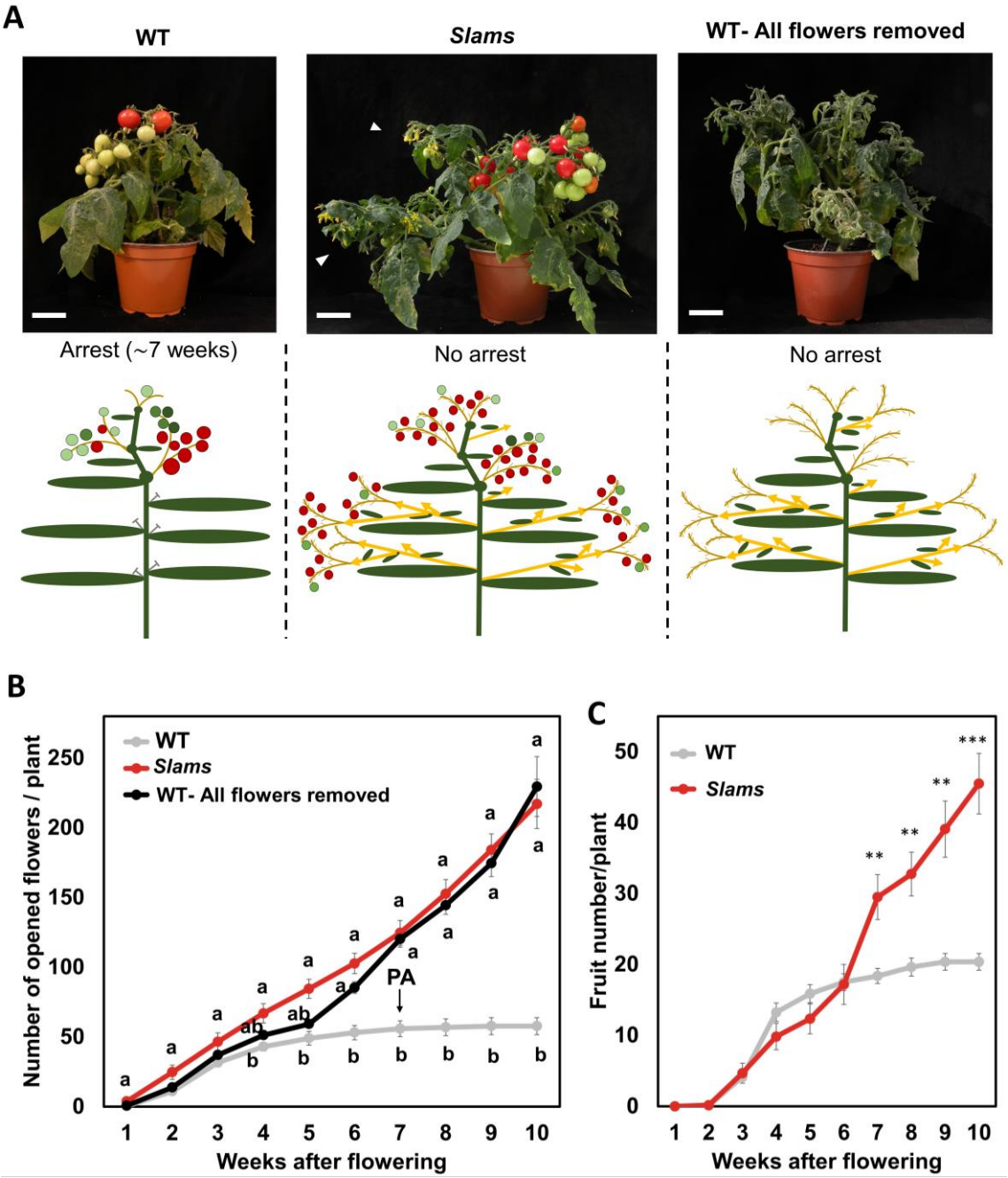


Figure 1.4. ***Slams* mutants do not undergo proliferative arrest. A)** Phenotype of wild type (WT), *Slams* mutant, and wild-type plants where all flowers were removed (WT-all). White arrows indicate axillary sympodial units with anthesis flowers. Scale bars= 5cm. **B)** Cumulative number of flowers opened during ten weeks of the reproductive phase. Different letters indicate significant differences within groups according to the *Kruskal-Wallis* test. **C)** Cumulative number of fruits produced per plant by the wild type and *Slams*. Asterisks indicate significant differences according to the Student's *t*-test *(**P<0.01*, ****P<0<001*). The mean of n=8 independent plants for WT and n=12 plants for *Slams* and WT-All. flowers removed is plotted. Error bars indicate the standard error of the mean (s.e.m).

Micro-Tom cultivar contains a mutation in the *TFL1*-ortholog *SELF-PRUNING* (*SP*) gene (Pnueli et al., 1998) that is highly expressed in axillary meristems (Thouet et al., 2008) and this mutation is required to maintain a determinate growth habit in tomato plants. In *sp* mutants, the number of leaves in subsequent sympodial units is gradually reduced until the production of two successive inflorescences and, thus, the growth of the main apex ceases. We used the cultivar Micro-Tom-*SP* (MT-*SP*) with indeterminate growth habit, to study the role of *SP* in the control of the end of flowering in tomato plants. In addition, we introduced the mutant allele of the *SlAMS* gene into this genetic background generating *Slams-SP* plants. The end of flowering occurred in the indeterminate MT-*SP* cultivar 12 weeks after the floral transition, while *Slams-SP* mutants also did not show proliferative arrest in this background and remained active indefinitely (Supplementary Fig. 1.S3).

These results suggest a role for *SP* in the regulation of the activity of the meristems, delaying proliferative arrest, but not preventing it. Again, in the *SP* background, seeds were the determinant factors as a source of signaling molecules triggering the end of flowering.

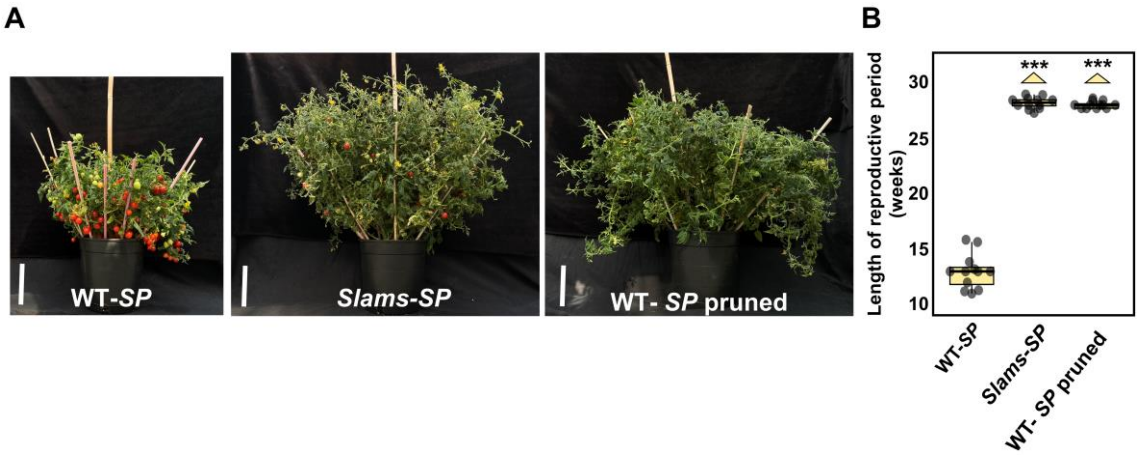


Figure 1.S3. ***Slams*** **mutants did not undergo proliferative arrest in Micro Tom *SP/SP* background.** A) Phenotype of the wild type (WT-*SP*), *Slams-SP*, and wild-type plants where all flowers were removed (WT-*SP* pruned). B) Length of reproductive period. The yellow arrow in the graph indicates that *Slams* mutants and pruned WT plants remain active and did not undergo proliferative arrest. Asterisks (*) indicate significant differences according to *Kruskal-Wallis* test. (****P < 0.001)*. Data correspond to n=12 independent plants. Scale bars = 15cm.

Role of phytohormones in the control of the end of flowering in tomato.

Previous studies proposed several hypotheses to explain the possible connection between fruit and seed development and the triggering of proliferative arrest. Some authors highlight the source-sink relations between developing fruits and the meristem or the existence of a mobile seed-derived signal that represses meristem activity and that promotes plant senescence, which was named 'death hormone' and whose nature is poorly understood (Engvild, 1989; Noodé & Penney, 2001; Ware et al., 2020; Wilson, 1997). Our characterization of *Slams* mutants supports this second scenario, and thus we decided to compare the phloem exudates from fertile and parthenocarpic seedless fruits of tomato.

We focused on the analysis of the phytohormone content comparing exudates from developing flowers at anthesis (0 DPA) and 4 fruit stages from early development (7 DPA) to mature fruits (48 DPA). Abscisic acid (ABA), jasmonic acid (JA), and auxin (IAA) could reliably be quantified in phloem exudates from fertile and seedless fruits. High levels of auxins were detected in the exudate of wild-type fruits at early stages of development (7 DPA and 15 DPA) and mature green stage (38 DPA), while they were very low in exudates of flowers in anthesis and fruits of the mature red stage (48 DPA). The auxin content measured in the exudates of seedless fruits did not fluctuate during fruit development and was up to 3.6 times lower than in wild-type fruits at 7 DPA, decreasing up to 4.7 times in fruits at 15 DPA. ABA and JA levels detected in wild-type fruit exudates showed a similar accumulation pattern. A maximum peak of ABA and JA was observed in immature green fruit at 7 DPA, quickly decreasing from immature green fruit at 15 DPA to mature red fruit (48 DPA). Seedless fruits did not show a peak of ABA or JA in exudates at early fruit development (7 DPA) or any of the stages analyzed (Fig. 1.5B-C). These results showed that developing fruits are a source of IAA, ABA, and JA that are loaded into the phloem and that the production of these molecules requires the presence of developing seeds. In connection with the absence of proliferative arrest in seedless tomato mutants, the production of a peak of these phytohormones at early stages of fruit development could be related to the signals that trigger proliferative arrest in fertile tomato plants.

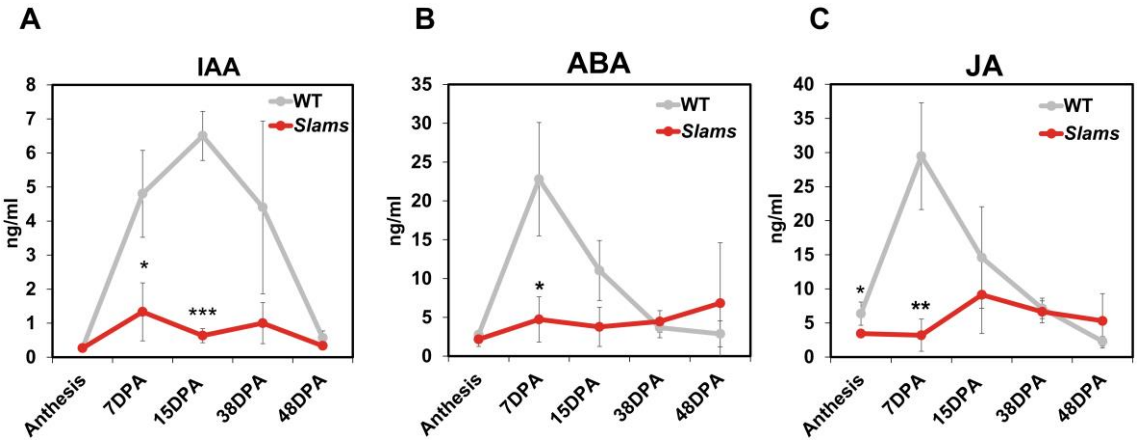


Figure 1.5. Analysis of phloem fruit exudates from wild-type and *Slams* mutant plants. Quantification of auxins **A)** abscisic acid B) and jasmonic acid C) in phloem fruit exudates from wild type (WT) and *Slams* at different stages of fruit development: Anthesis (0 DPA), 7 DPA, 15 DPA, 38 DPA, 48 DPA. Data correspond to the mean of 3 biological replicates ±SD. Asterisks indicate statistically significant differences according to Student's *t*-test (**P<0.05*;***P<0.01*;****P<0.001*). DPA= days post anthesis.

Exogenous auxin application to seedless fruit triggers proliferative arrest.

Hormone signaling pathways play a significant role in the integration of endogenous signals and environmental conditions to coordinate proliferative arrest. Recently, it has been reported that auxin exported from fertile fruits is an important component of the signaling mechanisms triggering proliferative arrest in Arabidopsis, specifically affecting its second component, floral arrest (Walker et al., 2023; Ware et al., 2020).

In tomato plants, a peak of auxins was detected in exudates of developing wild-type fruits (7 and 15 DPA) but not in those of seedless fruits (Fig. 1.5A). Previous studies in tomato indicate that auxins synthesized in seeds are transported through the fruit pedicel to the rest of the plant (Pattison & Catalá, 2012). Therefore, we hypothesized that auxins from fruits could play a key role in controlling proliferative arrest in tomato.

To test this hypothesis, we assessed whether the application of exogenous auxin to seedless fruit at early developmental stages could trigger the proliferative arrest of *Slams* mutant plants. 7 DPA seedless fruits from *Slams* plants which did not undergo proliferative arrest, were treated with the auxin agonist 1-naphthaleneacetic acid (NAA). *Slams* mutants showed a decrease in flower production rate after four weeks of treatment until proliferative arrest occurred at ~6-7 weeks. The end of flowering in the NAA-treated *Slams* mutant took place with the same timing as the wild type, while the untreated *Slams*

mutant did not arrest and continued producing flowers and fruits (Fig. 1.6A-B). Therefore, auxin treatment was very effective at inducing proliferative arrest in the seedless mutants. On the other hand, auxin treatment in *Slams* plants had no effect on fruit weight, which remained smaller than wild-type fruits (Supplementary Fig. 1.S4).

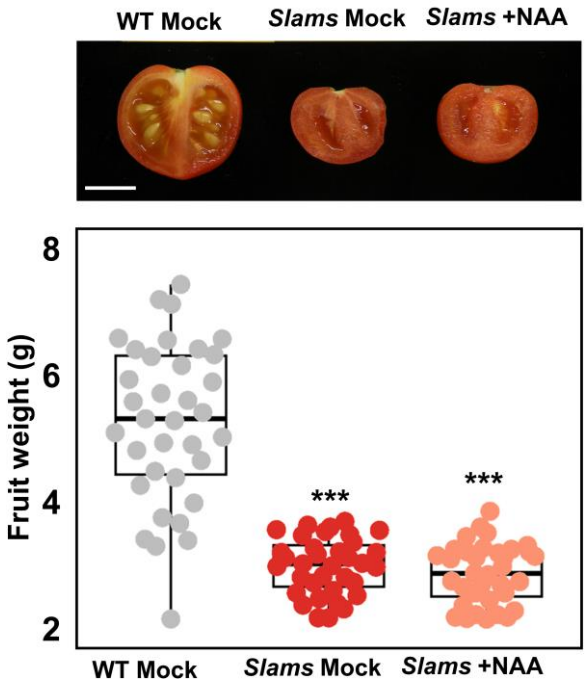


Figure 1.S4. Effect of NAA treatments on fruit weight. Ripe fruit weight of WT + Mock, *Slams* + Mock, and *Slams*+NAA plants. The mean weight of n=30 fruits/genotype is plotted. Asterisks indicate statistically significant differences according to Student's *t*-test (****P<0.001*). Scale bar= 1cm

Auxins could either exert their action directly or indirectly through the activation of the synthesis of a second messenger that triggers PA. The analyses of fruit exudates showed that seedless fruits produced a reduced amount of JA and ABA (Fig. 1.5B-C), suggesting that these two hormones could be also involved in proliferative arrest signaling together with auxins. Fruit-derived auxins could activate JA or ABA synthesis in the fruit, and subsequently, these hormones could be involved in signaling the meristems to arrest. We tested this hypothesis by analyzing JA and ABA content in exudates from NAA-treated seedless fruits. The application of NAA caused a 3.4-fold increase in ABA exuded from seedless fruit while there was no variation in the level of exuded JA in fruits treated with NAA (Fig. 1.6C-D). These results revealed a possible interaction of ABA and auxins in the control of proliferative arrest.

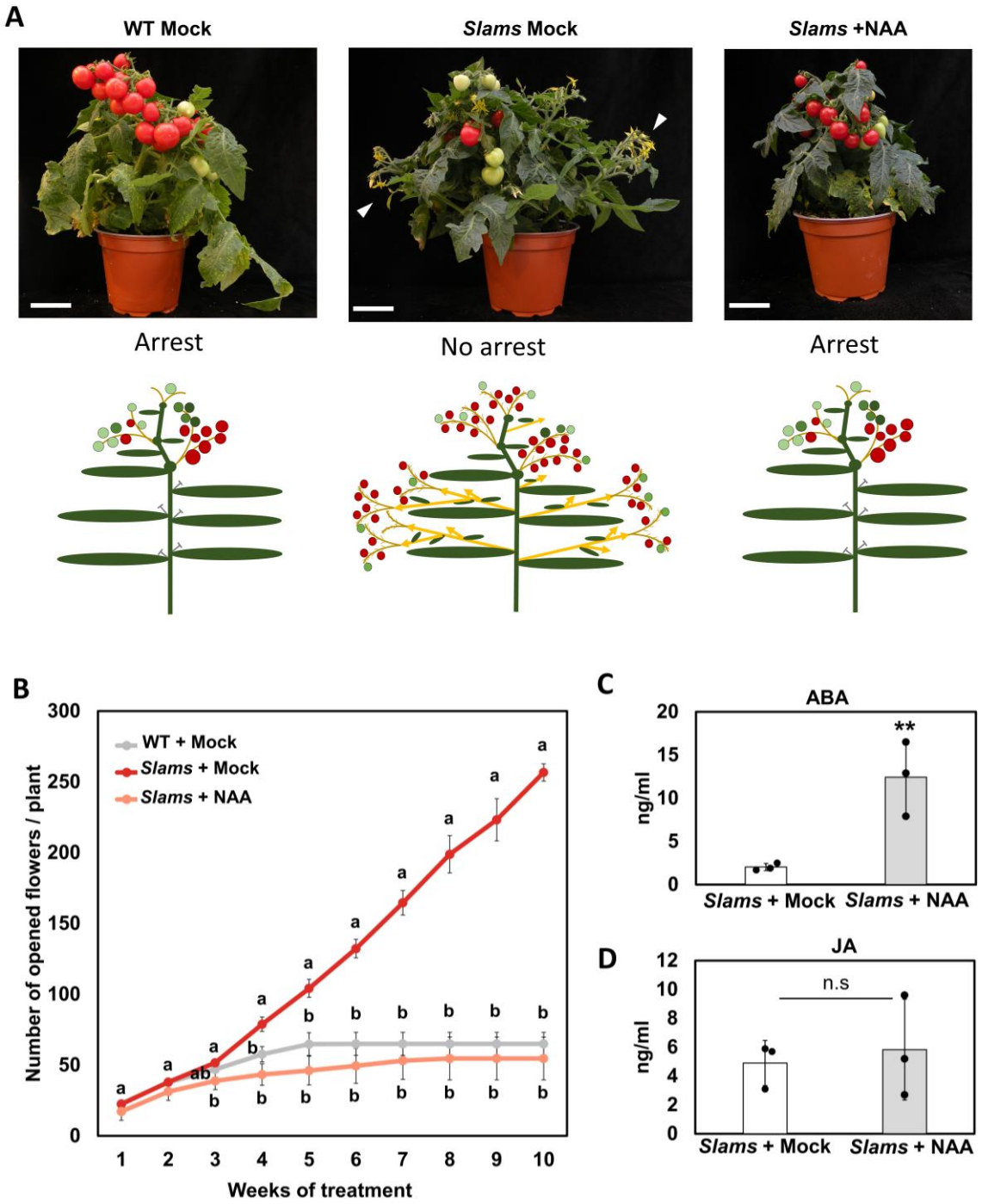


Figure 1.6. Application of exogenous auxin to developing fruits triggers proliferative arrest in *Slams* mutants. A) Phenotype of wild-type (WT) Mock, *Slams* Mock, and *Slams* + NAA plants seven weeks after treatment**.** Scale bars = 5cm. **B)** Number of flowers (cumulative) produced by WT Mock, *Slams* Mock, and *Slams* +NAA. Data show the mean of n=6 independent plants for WT + Mock and *Slams* + Mock and n=7 independent plants for *Slams* + NAA. Significant differences were inferred according to the one-way ANOVA test followed by the HSD Tukey post hoc test ($p<0.05$). Data sets with different letters indicate significant differences from each other. Error bars indicate the standard error of the mean (s.e.m). Quantification of ABA **C)** and JA **D)** in phloem fruit exudates from 7 DPA of

Slams+Mock and *Slams*+NAA plants. Asterisks indicate statistically significant differences according to Student's *t*- test (***P<0.01*).

The role of ABA in the control of the end of flowering was tested by treating seedless 7 DPA fruits with the ABA-receptor agonist, ABA mimicfluorine derivative 4 (AMF4; (Jiménez-Arias et al., 2023). AMF4-treated *Slams* mutants showed a decrease in flower and fruit production ratio compared to untreated mutants. Although mutant plants treated with the ABA agonist activated a lower number of lateral branches and consequently showed a reduction in the flower production rate, they had not undergone proliferative arrest at the end of the experiment (10 weeks) (Supplementary Fig. 1.S5 A-D).

Taken together, the results demonstrated that fruit-derived auxins play a major role in the control of proliferative arrest in tomato, triggering the global cessation of inflorescence activation. This effect could be at least in part mediated by promoting ABA synthesis and export from fruits, linked to the reduction of axillary sympodial growth.

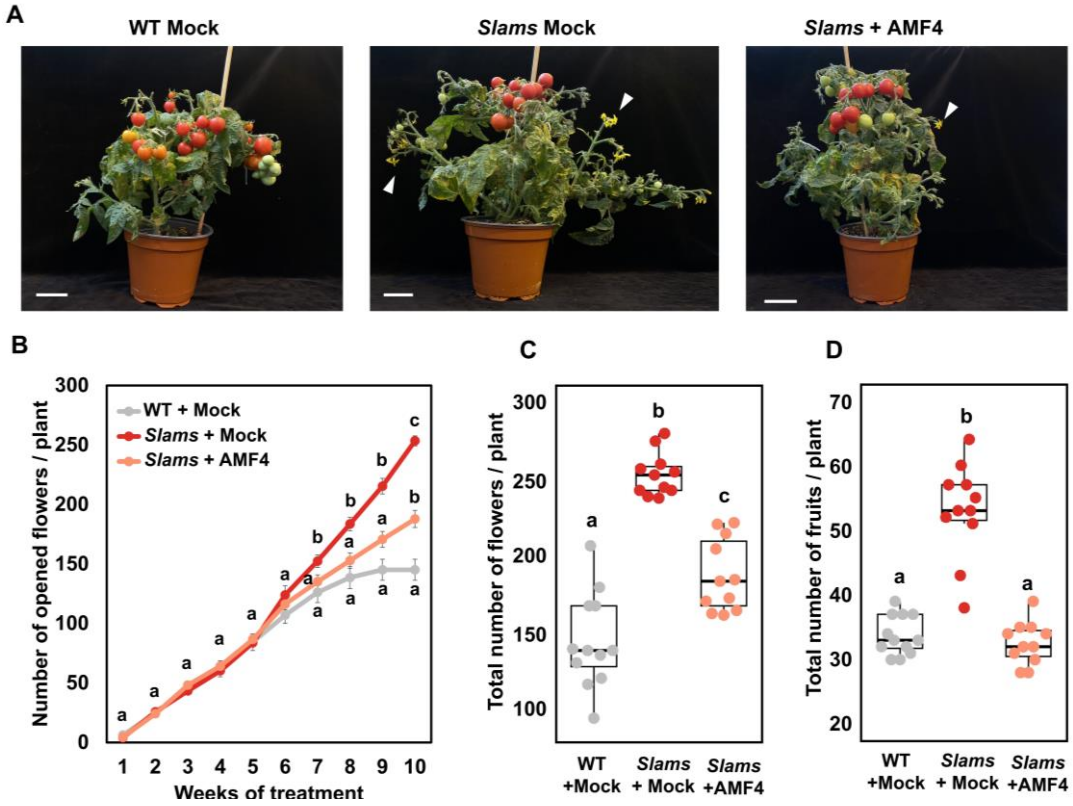


Figure 1.S5. Exogenous application of AFM4 to the fruits decreases the proliferative rate in the *Slams* mutants. A) Phenotype of WT Mock, *Slams* Mock, and *Slams* + AFM4 plants 10 weeks after treatment. White arrows indicate active developing lateral branches. **B)** Number of flowers (cumulative) produced by WT Mock, *Slams* Mock, and *Slams* + AFM4. **C)** Total number of flowers and **D)** fruits produced per plant in WT Mock, *Slams* Mock, and *Slams* + AMF4 at the end of treatment (10 weeks). Data correspond to n=12 total independent plants for WT + Mock and n=11 independent plants for *Slams* + Mock and *Slams* + AMF4. Significant differences were

inferred according to the one-way ANOVA test followed by the HSD Tukey post hoc test ($p<0.05$). Data sets with different letters at the top indicate significant differences from each other. Error bars indicate the standard error of the mean (s.e.m). Scale bars = 5 cm.

Discussion

In recent years, there has been renewed interest in understanding the regulation of flowering termination in monocarpic plants, particularly in the model organism *Arabidopsis thaliana*. Physiological, molecular, and genetic research is being integrated into an increasingly intricate framework to dissect this process (Balanzà et al., 2023; González-Suárez et al., 2020; Sadka et al., 2023). Nonetheless, more studies across other crop species are required to uncover shared and unique regulatory components, propose broader models, and support the development of widely applicable biotechnological approaches. In this study, we aim to contribute to this effort by performing a comparative analysis of the end of flowering in tomato, a particularly interesting crop that shows significant differences in inflorescence architecture and fruit development with respect to Arabidopsis. Our work demonstrates that the major influence triggering the end of flowering in tomato is seed production and supports the existence of a systemic signal that coordinately controls the production of new flowers by the plant.

Seeds as a Source of Signals Triggering Proliferative Arrest.

The generation and characterization of parthenocarpic seedless mutants of tomato that do not experience proliferative arrest has allowed us to unequivocally conclude that the seeds, rather than the fruits, are the most relevant factor that instructs the plant to stop forming new flowers. These findings challenge the idea that the sink strength of the developing fruits deprives the meristems of resources (Durán-Soria et al., 2020; Gifford & Evans, 1981; L.C.Ho, 1996) and is then the cause of proliferative arrest.

We have determined in untreated and partially defruited plants that the production of a certain number of fruits with seeds is required to arrest meristem activity coordinately in all plant positions, and that if after this point fruits are eliminated, dormant axillary meristems become active and resume flower and fruit production until this critical number is reached again. This global and quantitative nature of the seed effect strongly supports the hypothesis of seeds as the source of a systemic signal that causes the cessation of flower production.

Our results are in line with a recent parallel study in pea (*Pisum sativum*) that also shows this quantitative and systemic effect of seed production on proliferative arrest (Burillo et

al., 2024). These similarities are particularly interesting given the differences in fruit morphology and physiology of tomato and pea: pea plants develop dry dehiscent fruits, fully photosynthetic throughout development, while tomato plants form fleshy berries that accumulate sugars and other compounds as they ripen, being fully heterotrophic and therefore with a much stronger sink capacity. Interestingly, in Arabidopsis, despite being more similar to pea in developing dry dehiscent fruits, proliferative arrest does not appear to respond in such a quantitative manner to the number of seeds produced, taking place after the production of a similar number of flowering nodes, even in plants with a wide range of seed production (Hensel et al., 1994). This could be explained if either developing fruits and not only seeds, contribute to the production of the arrest-triggering signal in Arabidopsis or if additional factors, such as the age of the inflorescence or other unknown elements, have more relevance in the process in this species. These hypotheses will remain to be tested until the nature and mechanism of action of the arrest signal is uncovered.

Hormones are part of the signaling mechanism triggering arrest

We have shown that tomato fertile fruits export auxins, ABA and JA, likely produced by the developing seeds, since these hormones are detected at much lower levels in parthenocarpic fruit exudates.

Indole-3-acetic acid (IAA) is the main endogenous auxin, and the concentration of this hormone is high in tomato seeds (Pattison & Catalá, 2012; Varga & Bruinsma, 1976). It has been proposed that the seeds serve as a source of auxin that is transported from the fruit to the parent plant through the pedicel, and this process appears to prevent premature fruit abscission (Bangerth, 2000; Banuelos et al., 1987; Serrani et al., 2010). Here, we show that auxin exported from seeds could also be an important component of the mechanisms controlling the end of flowering, since when seedless parthenocarpic fruits were treated exogenously with this hormone, they were able to trigger proliferative arrest as efficiently as fertile fruits. Interestingly, auxin treatment of parthenocarpic fruits also promotes the accumulation of ABA, but not JA in the exudates. While this discards JA as an essential component of the signals between seeds and meristems leading to flowering cessation, the putative role of ABA in the process is strongly suggested. In fact, exogenous treatment of parthenocarpic fruits with AMF4, an ABA agonist (Jiménez-Arias et al., 2023), caused a significant reduction of meristem activity and the rate of floral production, although did not trigger proliferative arrest as auxin did. These results indicate that ABA could mediate, at least partially, the auxin effect on proliferative arrest.

However, because we have used AMF4, an agonist that overcomes the rapid loss of ABA bioactivity under UV-light but shows some differences in affinity and activity to PYR/PYL receptors than ABA (Jiménez-Arias et al., 2023), we cannot rule out that endogenous ABA production by seeds may have a stronger effect on meristem arrest, similar to that of auxin.

The sympodial nature of tomato plant architecture and the mode of proliferative arrest

The role of auxin and ABA in the regulation of proliferative arrest has been previously described in *Arabidopsis*. At this point, it is necessary to stress the differences in plant architecture between Arabidopsis and tomato. Arabidopsis has a monopodial growth habit. Once the floral transition has occurred, the shoot apical meristem acquires inflorescence meristem identity, producing flowers at its flanks. Proliferative arrest in this species has two components: first, inflorescence meristem arrest, when IM becomes inactive, and second, floral arrest when unpollinated floral buds enter a developmental block. Both auxin and ABA roles in proliferative arrest have been restricted by previous studies to this second component and do not appear to influence the timing of IM arrest (Sánchez-Gerschon et al., 2024; Walker et al., 2023). In contrast, tomato plants bear flowers in inflorescence meristems that are determinate. Therefore, plant growth and flower production does not depend on the maintenance of the activity of the inflorescence meristems, but on the production of new sympodial units by activation of axillary meristems. Then, it is possible that proliferative arrest only requires the developmental block of these axillary meristems, and thus, the role of auxin and ABA could be more prominent in tomato than in Arabidopsis.

Our work also shows how seed production affects globally the activation of new inflorescence meristems, regardless of their relative position with respect to the main apex, both in *sp* and *SP* backgrounds. Removing all fruits from the developed sympodial units of arrested plants reactivates dormant axillary meristems. This reactivation implies a systemic coordination of meristem activity, where a critical threshold of fruits and seeds across all sympodial units induces a global proliferative arrest. This coordinated and synchronized cessation of meristem activity across sympodial meristems is similar but not fully equivalent in *Arabidopsis*, where the end of flowering has been proposed to result primarily from an uncoordinated, local arrest of individual inflorescences (Ware et al., 2020)(Ware et al., 2020), and the fruits proximal to the shoot apical meristem are more effective in triggering arrest, while those in lateral branches play a lesser role. Again, the indeterminate character of the *Arabidopsis* inflorescence meristem could require additional components to restrict its activity with a stronger local component.

Conclusion

In summary, our study provides important insights into the hormonal and physiological mechanisms controlling proliferative arrest in tomato, a globally synchronized process likely systemically controlled. We also identify the seeds as the major factor triggering arrest, where hormones like auxin and ABA play a critical role. These results not only expand our understanding of how crop plants regulate reproductive development, but also provide a framework for future studies aimed at improving yield and optimizing the timing of fruit production in tomato and other monocarpic species.

Materials and Methods

Plant material and growth conditions.

Tomato (*S. lycopersicum L*.) seeds from Micro-Tom and Micro-Tom (*SP/SP*) that show "determinate" and "indeterminate" growth, respectively, were used as wild-type plants. In the greenhouse, plants (one per pot) were grown in 12cm and 17 cm pots, respectively, with a mixture of peat: perlite (1:1 v/v) in 24/20 ºC day/night conditions and irrigated with Hoagland's nutrient solution (Hoagland & Arnon, 1950). Natural light was supplemented with Osram lamps (150 µmol m^{-2} s^{-1}, Powerstar HQI-BT, 400W) when required to get a 16h light/8h dark photoperiod.

Plasmid assembly for *SlAMS* gene editing

To target the *SlAMS* gene, Breaking-Cas web tool (Oliveros et al., 2016) was used to design a single gRNA with the highest score and the lowest number of off-target genes. To assemble the CRISPR/Cas9 construct we used the GoldenBraid (GB) modular toolbox (Vazquez-Vilar et al., 2016). First, we adapted the 20nt target sequence to the GB system using with the 'GB-CRISPR domesticator' tool and then it was assembled with GB1001 (U626 promoter) and GB0645 (scaffold RNA) parts into the destination vector pDGB3α1 to create the guide RNA expression cassette. In sequential GB reactions, this cassette was assembled with *hCas9* (GB0639) and *nptII* (GB1181) transcriptional units into a destination vector. *Agrobacterium tumefaciens* strain LBA4404 was transformed with the final CRISPR/Cas9 construct to then proceed to plant transformation.

Plant transformation and genotyping of CRISPR/Cas9 edited plants

To obtain tomato transformants, tomato cotyledons were co-cultured with *Agrobacterium tumefaciens* strain LBA4404 with the binary vector containing the construction of interest (Ellul et al., 2003). Regenerated transformant plants were selected and rooted in the presence of kanamycin and acclimated in the greenhouse for further analysis.

To identify the type and percentage of edition in the transformant plants, genomic DNA was extracted from young leaves, and a 592 bp fragment flanking the targeted sequence was amplified by PCR using SlAMS For/Rev primers (Supplementary Table 1.S1). These fragments were purified and sequenced, and the presence of mutations was analyzed by sequence trace decomposition using the online tools: TiDE (http://shinyapps.datacurators.nl/tide/) (Brinkman et al., 2014) and ICE 2 CRISPR (https://ice.synthego.com). *Slams* mutants were male sterile. T0 generation was pollinated with wild-type pollen and Cas9-free heterozygous plants were selected in T1 generation.

We designed a derived cleaved amplified polymorphic sequence (dCAPS) marker to genotype homozygous edited plants (http://helix.wustl.edu/dcaps/) (Neff et al., 2002)(Neff et al., 2002). A 277-bp fragment was obtained from genomic DNA with the dSlams For/Rev primers (Supplementary Table 1.S1) generating a restriction site for EcoRI in the *Slams* mutant sequence. EcoRI digestion generated two fragments of 249bp and 28bp in the mutant sequence. The wild-type sequence is not recognized by this restriction enzyme.

Micro-Tom cultivar was transformed, and after the identification of *Slams* mutant alleles, they were introduced into the Micro-Tom *SP/SP* cultivar by crosses.

Table 1.S1- Primers used in this study.

Oligo name	Sequence (5'->3')	Gene ID	Experiment
SlAMS CRP for	ATTGCTCTATGCTCTTAGAGCTT	Solyc08g062780	CRISPR guide *SlAMS*
SlAMS CRP rev	AAACAAGCTCTAAGAGCATAGAG		
SlAMSG For	TCAAGCTGGAATTGATGATGA	Solyc08g062780	*SlAMS* edition analysis
SlAMSG Rev	TGAATCTGGTCCGAATTCCT		
dSlAMS For	CCTTGGAGATTTTGGGAACCGAAT	Solyc08g062780	*Slams* mutant genotyping
dSlAMS Rev	GGAACCTCTAAAACCCTTGGCTTA		

Phenotypic analysis

The number of open flowers per plant was measured from the first anthesis flower developed until the last flower opened at the time of the end of flowering. Plants that did not arrest (*Slams* and WT-pruned) were monitored on average for 10 weeks on the determinate Micro-Tom cv. and for 28 weeks on the indeterminate Micro-Tom cv. (*SP/SP*). The total number of open flowers and new fruit was quantified weekly. The length of the reproductive period was established as the time from the first flower at anthesis to the last open flower developed. For the analysis of tomato fruits were measured the weight and number of seeds of at least 20 ripe fruits of the different genotypes.

Histological analysis

Flower samples were collected at different stages according to bud size (Brukhin et al., 2003). Four flower stages were analyzed that correspond to the following bud sizes: stage 8 (0.2 cm), stage 10 (0.3 cm), stage 14 (0.4 cm), stage 16 (0.6 cm). Tissue was fixed in FAE (3.7% formaldehyde, 5% acetic acid, 50% ethanol) overnight at 4ºC and stored in 70% ethanol. Samples were dehydrated through an ethanol series (85% to100%) and embedded in acrylic resin (Technovit 7100; Kulzer) according to the manufacturer's instructions. For histological analysis, resin sections (~2 µm) were stained with 0.02% toluidine blue in 0.1 M, pH 6.8, phosphate buffer (O'Brien et al., 1964) and visualized under bright field with a Leica DM 5000B microscope (Leica Microsystems).

Phloem fruit exudates collection and phytohormone quantification.

For the quantification of phytohormones (IAA, JA, ABA) anthesis flowers from the first inflorescences of Micro-Tom cv were marked. Fruits at different stages of development were collected: immature green (7 DPA, 15 DPA), mature green (28 DPA), and mature (48DPA). Individual fruits were placed pedicel-down in a 96-well plate containing 250 uL of 5 mM EDTA buffer to prevent sealing of the phloem and incubated for 19 h in the dark in a growth chamber. Samples were frozen in liquid nitrogen and freeze-dried for further analysis. Three biological replicates containing phloem exudate from n=3 fruits were used for each developmental stage.

For the quantification and extraction of plant hormones, freeze-dried samples were suspended in 80% methanol-1% acetic acid containing internal standards and mixed

by shaking one hour at 4ºC. The extract was kept at -20ºC overnight and then centrifuged and the supernatant dried in a vacuum evaporator. The dry residue was dissolved in 1% acetic acid and passed through an Oasis HLB (reverse phase) column as described in (Seo et al., 2011).

For IAA, ABA and JA quantification, the dried eluate was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an autosampler and reverse phase UHPLC chromatography (2.6 µm Accucore RP-MS column, 100 mm length x 2.1 mm i.d.; ThermoFisher Scientific) with a 5 to 50% acetonitrile gradient containing 0.05% acetic acid, at 400 µL/min over 21 min. The hormones were analyzed with a Q-Exactive mass spectrometer (Orbitrap detector; Thermo Fisher Scientific) by targeted Selected Ion Monitoring (SIM). The concentrations of hormones in the extracts were determined using embedded calibration curves and the Xcalibur 4.0 and TraceFinder 4.1 SP1 programs. The internal standards for quantification of each of the different plant hormones were the deuterium-labeled hormones, except for JA, for which the compound dhJA was used.

Hormone treatments.

The treatment of 200 ng/µl (1mM) of 1-naphthaleneacetic acid (NAA) and 44.45 ng/µl (100 µM) of ABA mimicfluorine derivative 4 (AMF4) was applied in 10 µL of solution to 7 days post anthesis (DPA) fruit. The mock solution contained 5% ethanol and 0.1% Tween 80 for NAA and 0.1% DMSO, 0.1% Tween 80, and 10 mM MES pH 5.7 for AMF4 treatment. Treatments started at 7 DPA from the first flower until the last 7 DPA fruit developed from the last flower, when plants underwent proliferative arrest.

The treatment of 200 ng/µl (1mM) of 1-naphthaleneacetic acid (NAA) and 44.45 ng/µl (100 µM) of ABA mimicfluorine derivative 4 (AMF4) was applied in 10 µL of solution to 7 days post anthesis (DPA) fruit. Mock solution contained 5% ethanol and 0.1% Tween 80 for NAA and 0.1% DMSO, 0.1% Tween 80 and 10 mM MES pH 5.7 for AMF4 treatment. Treatments started at 7 DPA from the first flower until the last 7 DPA fruit developed from the last flower, when plants undergo proliferative arrest.

Determination of ºBrix index

To measure ºBrix, five ripe fruits per plant were collected from nine independent plants of both genotypes, wild-type Micro-Tom and *Slams* mutant. Epidermis and seeds were removed before grinding the fruits to obtain a filtrate. ºBrix index was determined with

the ATAGO N-14 refractometer. Three biological replicates per genotype containing the filtrate of fifteen ripe fruits each were analyzed.

Statistical analysis

Shapiro-Wilk normality test was used to evaluate the normal distribution of the data sets. Normally distributed data were analyzed with a Student's t-test. Non-normally distributed data were analyzed with the Kruskal-Wallis test. For the statistical analysis, IBM SPSS Statistics 26 was used (IBM, https://www.ibm.com).

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Conflict of interest

None declared

References

References of chapter 1 are listed at the end of the thesis manuscript.

Supplementary material.

The supplementary material of Tables (1.S1) and Figures (1.S2-S5) are placed in the corresponding sections in the thesis manuscript to facilitate the interpretation of the results.

Chapter 2

Genetic control of proliferative arrest in tomato by the FUL-AP2-miR172 module.

Introduction

The end of flowering in monocarpic plants takes place after the production of a certain number of fruits, acting as a mechanism to ensure that plants allocate their resources efficiently to maximize fruit filling, seed production, and, therefore, reproductive success. This process, called "Proliferative Arrest" (PA), is largely influenced by specific signals from fruits and seeds, which are major factors in controlling PA in monocarpic species, including crops such as tomato (Murneek, 1926) and *Pisum sativum* (Burillo et al., 2024; Lockhart & Gottschall, 1961). In addition to the effect of fruits/seeds, other endogenous and environmental factors also contribute to modulate the timing of PA (Balanzà et al., 2023; González-Suárez et al., 2023; Martínez-Fernández et al., 2020).

In recent years, substantial progress has been made in understanding the genetic and molecular mechanisms regulating proliferative arrest (PA) in *Arabidopsis thaliana* (Balanzà et al., 2023; Sadka et al., 2023). The first genetic factors involved in the control of PA have been identified in this species, organized in a small regulatory module that regulates inflorescence meristem activity. The module involves the transcription factors *FRUITFULL* (*FUL*) and *APETALA2* (*AP2*) the latter being regulated by miRNA172. (Balanzà et al., 2018). This pathway would act in parallel to the seed-derived signals that also promote PA (Balanzà et al., 2018; Hensel et al., 1994; Ware et al., 2020).

Proliferative arrest in Arabidopsis, a plant with monopodial growth habit and a simple raceme-type inflorescence, involves the cessation of the production of new primordia by the inflorescence meristem. *WUSCHEL* (*WUS*) is the main factor that ensures the maintenance of the stem cell niche and thus supports meristem activity. Accordingly, *WUS* expression is no longer detected in the inflorescence meristem at the moment of PA (Balanza 208, Merelo 2022). AP2 promotes *WUS* maintenance in the meristem, likely in a redundant manner with other members of the AP2 family (Würschum et al., 2006). As plant ages, *AP2* expression is, in turn, repressed by FUL, a MADS-box transcription factor with multiple roles in development, and at post-transcriptional level by miR172 (Martínez-Fernández et al., 2020; Zhu & Helliwell, 2011b). Throughout the reproductive cycle, the expression of *FUL* and miR172 increases in the inflorescence, leading to gradual repression of *AP2/AP2-like* genes in the shoot apical meristem (J. W. Wang et al., 2009; Wu et al., 2009) and the concomitant extinction of WUS expression, associated

with the cessation of meristem activity. Further work has shown that both FUL and AP2 factors also regulate directly several hormonal pathways in the meristem, such as cytokinin and ABA signaling, contributing to the transcriptional reprogramming of meristem activity at PA (Balanzà et al., 2018; Martínez-Fernández et al., 2020; Merelo et al., 2022; Sánchez-Gerschon et al., 2024). These works also hint at the possibility that *AP2-like* genes could integrate some environmental and seed-derived signals to modulate PA (Balanzà et al., 2023; Martínez-Fernández et al., 2020), highlighting the central role of the FUL-AP2-miR172 module in the regulation of the end of flowering in Arabidopsis.

In tomato, eight homologs of the Arabidopsis *AP2* gene have been identified as *SlAP2a* to *SlAP2e* (Karlova et al., 2011; Sun et al., 2023), in addition to several *AP2-like* genes (Chung et al., 2010). The expression of *SlAP2/SlAP2-like* genes in tomato is tissue- and developmental-stage-dependent (Bemer et al., 2012; Karlova et al., 2011). *SlAP2b* shows a more specific expression in flowers, while *SlAP2c* also has a predominant expression in flowers as well as in early stages of fruit development. In contrast, *SlAP2a* and *SlAP2d* appear to control fruit development at early and late stages, respectively (Chung et al., 2010). This pattern of expression suggests a specialized role for *SlAP2a* and *SlAP2d* in controlling tomato fleshy fruit development. *SlAP2e*, however, is expressed to a greater extent in vegetative tissues such as leaves and roots, with minimal expression in flowers or fruits (Karlova et al., 2011). Among *SlAP2-like* genes, *TARGET OF EAT1* (*SlTOE1*) is predominantly expressed in flowers and inflorescence meristems (IM), and in the floral meristem (FM). *SlTOE1* acts as a suppressor of inflorescence branching and regulates flower and inflorescence development by binding to and repressing transcription of *STM3* in FM (Sun et al., 2023). *SlTOE2*, a homolog of *SlTOE1*, is mainly expressed in the transitional meristem (TM), suggesting a role in the regulation of flowering time.

miRNA172 targets the members of the *AP2/AP2-like* family of transcription factors, leading to their repression either by mRNA degradation or translational inhibition. The mature sequences of miRNA172 are identical between *Arabidopsis thaliana* and *Solanum lycopersicum*, with only slight variations in the terminal nucleotides. Therefore, it is likely that miRNA172 regulates *SlAP2/SlAP2-like* genes also in tomato, influencing meristem activity and the timing of flowering termination(Karlova et al., 2011).

Regarding the tomato AP1/FUL-like genes, they are part of the MADS-box family of transcription factors, and several members of this family are known to regulate developmental processes in plants, including the induction of flowering and the

specification of the floral meristem (Jiang et al., 2022). These genes are divided into three primary clades: euAP1, euFULI, and euFULII (also known as AGL79-like) (Litt & Irish, 2003) (Figure 2.1A). This division is consistent with previous findings in central eudicots, where AP1/FUL genes play essential roles in flowering and fruit development (Berbel et al., 2012; Ferrándiz et al., 2000; Litt & Irish, 2003). Tomato has undergone lineage-specific duplications, giving rise to two paralogues in the euFULI and euFULII clades (*FUL1*, *FUL2*, and *MBP10*, *MBP20*, respectively). This suggests possible subfunctionalization during tomato evolution, where each paralog may have retained part of the functions of the original gene (Jaakola et al., 2002; Pabón-Mora et al., 2013; Ping et al., 2014).

Despite the increasing knowledge of the genetic networks controlling the end of flowering in Arabidopsis, the factors controlling proliferative arrest in other monocarpic plant species are poorly understood and the functional conservation of the FUL-AP2-miR172 module in PA regulation remains to be studied. A recent study has revealed that in *Pisum sativum*, the euFUL I genes *PsFULa* and *PsFULb* have a similar role in PA to the Arabidopsis homolog. *psfula* and *psfulb* mutants show a longer reproductive period, leading to an increase in the number of reproductive nodes and fruits than the wild type (Martínez-Fernández et al., 2020). This suggests that at least some elements of the FUL-mediated regulatory pathway may be conserved in different eudicot species.

To assess the functional conservation of the FUL-AP2-miRNA172 genetic pathway, we chose tomato, a crop species with big differences in terms of plant and inflorescence architecture that develops fleshy fruits. We studied the role of *SlAP2/SlAP2-like* genes by obtaining plants with modified levels of miR172, a negative regulator of *SlAP2* expression. The role of the tomato *FUL* genes in PA was studied by specifically targeting *MBP20* to generate knockout plants.

Results

Expression analysis of *SlFUL* genes during primary shoot maturation in tomato.

At the time this thesis was started, several studies reported that the euFULI clade genes *FUL1* and *FUL2* were involved in fruit development and maturation in *Solanum lycopersicum* (Bemer et al., 2012; Shima et al., 2013; R. Wang et al., 2019). Later, and parallel to our work, it has been shown that *FUL1*, *FUL2,* and *MBP20* have a role in the regulation of meristem dynamics throughout the vegetative and reproductive phases, controlling flowering time and inflorescence branching, whereas *MBP10,* with an unclear functional role, is considered a pseudogene (Park et al., 2012). In any case, no phenotypes related to proliferative arrest have been previously described for any of the mutants in *SlFUL* genes.

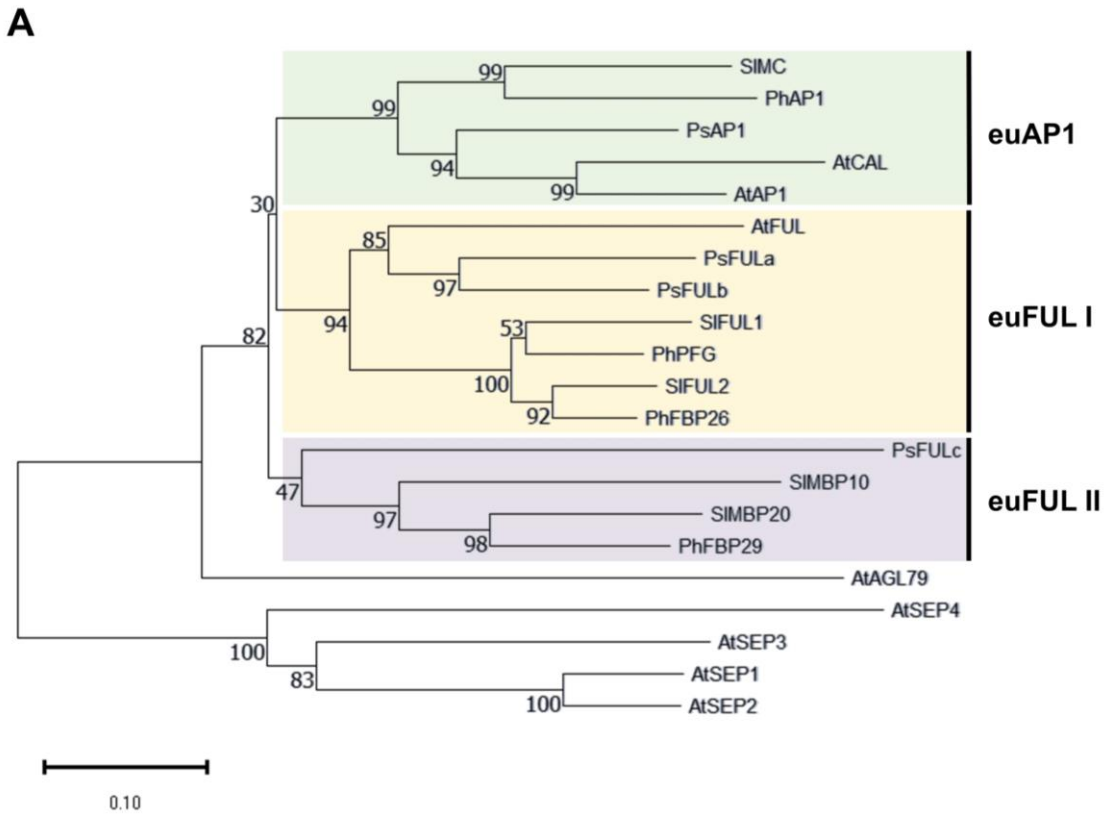
The expression pattern of *SlFUL* genes has been described from transcriptomic data in which *FUL1* and *FUL2*, but not *MBP20* are found in the FMs (Park et al., 2012). A later study, by Jiang et al.,2022 shows that the expression of the three genes was high in IM/FM samples (collected together), showing *MBP20* the highest level of expression. Combining these two studies, it could be deduced that *MBP20* was mostly associated with the IM and, therefore, a good candidate to control the activity of this meristem.

To better characterize the spatial and temporal expression patterns of *MBP20*, *FUL1*, and *FUL2* during meristem maturation and floral transition, we performed RNA *in situ* hybridization on the primary shoot meristem (PSM) at different developmental stages. Apical shoots collected 6 days after germination (DAG) correspond to a SAM at the vegetative stage. Apices ten days after germination (10 DAG) correspond to the reproductive stage after the floral transition and contain the inflorescent meristem (IM) and floral meristem (FM) together with the developing sympodial shoot meristem (SYM). Finally, at 13 DAG the shoot apices contain developing flowers (F), a higher number of IMs, and advanced sympodial meristems (Figure 2.1.).

At 6 DAG, before floral transition, *MBP20* expression was weakly detected in the vegetative shoot apical meristem (SAM) and leaf primordium (Lf) (Fig 2.1B-C). In addition, a hybridization signal was detected in the axillary meristem (AM), suggesting that *MBP20* could have an early role in regulating axillary meristem activity. A similar expression pattern was observed for *FUL1* and *FUL2* at this stage (Fig 2.1G-H and L-M). At about 10 DAG, *MBP20* was still expressed in leaf primordia, but its presence was also observed in the floral meristem (FM), inflorescence meristem (IM) and sympodial

meristem (SYM) just after the floral transition (Fig 2.1D-E). We also found *MBP20* transcripts in the axillary meristem during the reproductive phase, suggesting its involvement in both vegetative and reproductive growth. Similarly, *FUL1* and *FUL2* were expressed in the FM, IM, and SYM at this point (Fig 2.1I-J, N-O). At late reproductive stages, *MBP20* remained active in the axillary meristems but was absent from flowers (Fig 2.1F). In contrast, *SlFUL2* and *FUL1* maintained their expression in flowers and axillary meristems (Fig 2.1J-K, O-P), showing their continued involvement in meristem maturation.

These results show that the expression pattern of *FUL1* and *FUL2* is compatible with a role in the regulation of meristem dynamics throughout the vegetative and reproductive phases. *MBP20* seems to have a more specific function at later stages of reproductive development, where it was confined to the SYM and axillary meristems. The persistent expression of the three genes in axillary meristems also suggests that they could be regulating the transition from active arrested meristems at the end of the reproductive phase.



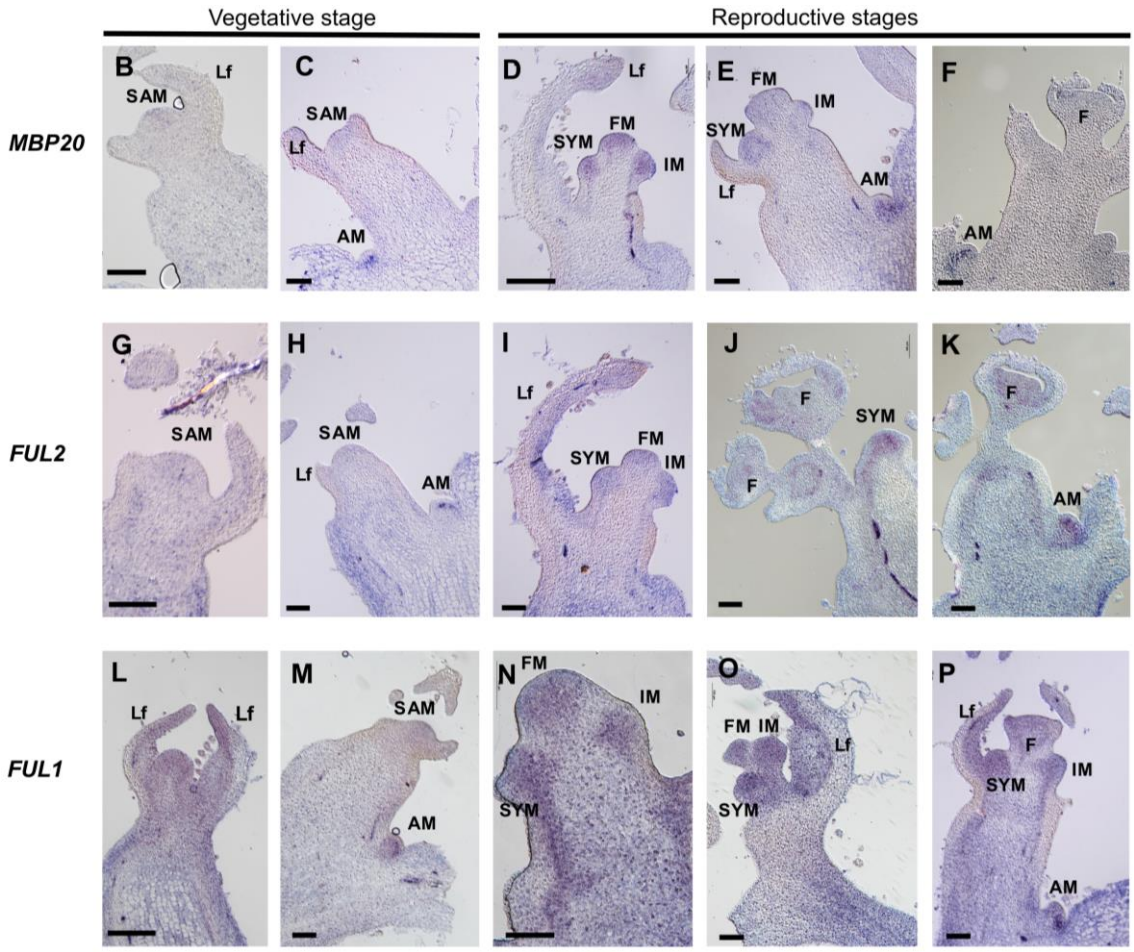


Figure 2.1. Phylogenetic analysis and expression of *SlFUL* genes during primary shoot meristem maturation in tomato. A) Phylogenetic analysis of *AP1/FUL*-like genes of *A. thaliana* (*At*), *S.lycopersicum* (*Sl*), *P. hybrida* (*Ph*), and *P.sativum* (*Ps*) The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is shown to scale, and the branch lengths correspond to the evolutionary distances used to estimate the phylogenetic tree. **B-O)** *MBP20, FUL2, and FUL1* expression analyzed by *in situ* hybridization in shoot apices from vegetative and reproductive stages**. B-C)** Longitudinal section of 6 DAG tomato seedlings showing weak *MBP20* expression in the vegetative SAM and leaf primordia (Lf). Also, *MBP20* mRNA was detected in the axillary meristem (AM). **G-H)** *FUL2* and *FUL1* **(L-M)** mRNA showed an analogous expression pattern. **D-E)** Longitudinal sections of tomato seedlings. *MBP20* keep its expression in the leaf primordia, and after floral transition its accumulates in the floral meristem (FM), in the inflorescence meristem (IM) and sympodial meristem (SYM). Also, it was detected in the axillary meristem (AM) in this stage. **I-J)** *FUL2* accumulates in the FM, IM, and SYM together with FUL1 **(N-O). F)** *MBP20* keeps its expression in the axillary meristem at later reproductive stages of the primary shoot meristem and residual expression in flower (F). **J-K)** FUL2 and FUL1 (**O-P**) mRNA maintains its expression in flowers and in axillary meristems at late stages of reproductive development. Bar in B, D, G, L and N indicate 100 µm. Bars in C, E, F, H, I , J, K, M, Ñ and O indicate 50 µm. The stages shown (vegetative and reproductive) correspond to 6, 10 and 13 DAG plants.

Potential functional divergence among FUL homologs was assessed by comparing the amino acid sequences of tomato FUL-like proteins (FUL1, FUL2, MBP10, and MBP20) with the Arabidopsis FUL (AtFUL1) protein. Sequence alignments reveal conserved and variable regions, with specific amino acid differences highlighted (Supplementary Fig. 2.S.1). These differences are found in key regions that may influence the structural or functional properties of each protein. FUL1 and FUL2 align closely with AtFUL1, suggesting a conserved function. In contrast, MBP20 and MBP10, which in the phylogenetic tree group located in the euFULII clade, exhibit unique variations in these highlighted regions, which may indicate a divergent function or specialization. The sequence divergence in certain regions of MBP20 could support the hypothesis that it has acquired different functions to FUL1 and FUL2, also consistent with its distinct expression pattern observed in *in situ* hybridization analyses (Fig 2.1).

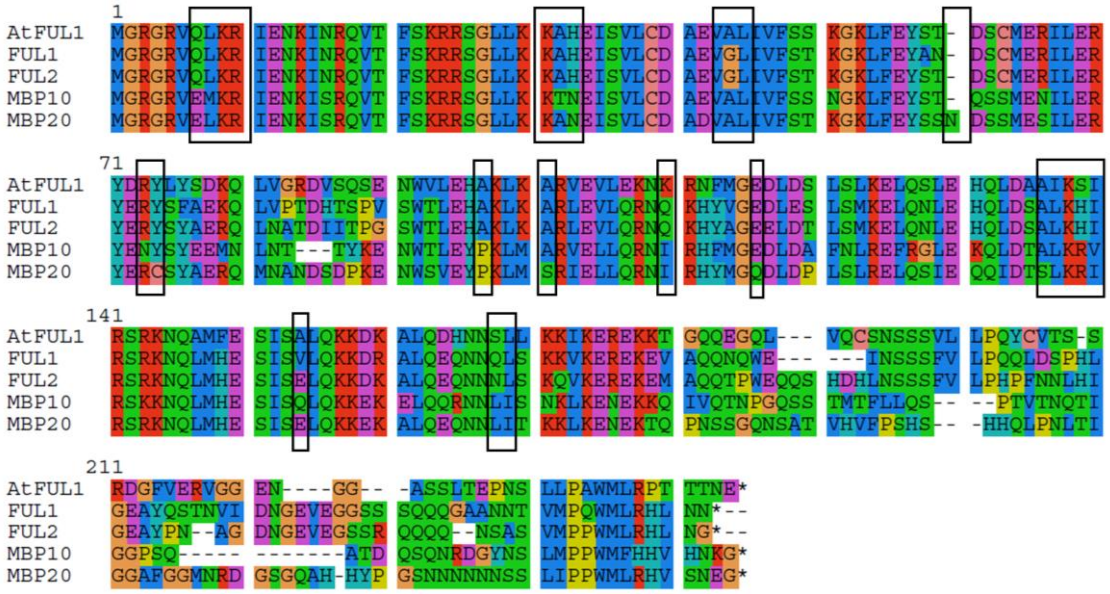


Figure 2.S.1. Sequence homology analysis of the Arabidopsis FUL (AtFUL1) protein and tomato FUL-like proteins (FUL1, FUL2, MBP10, and MBP20). Highlighted regions indicate amino acid sequence differences among the FUL proteins analyzed, suggesting areas of potential functional divergence.

MBP20 controls the end of flowering, fruit size and axillary branching in tomato plants

Previous studies in *Arabidopsis thaliana* and *Pisum sativum* have shown loss-of-function mutations in euFUL I genes such as *AtFUL* or *PsFULa* and *PsFULb* lead to a delayed proliferative arrest (PA), resulting in an extended reproductive phase. In both species, *ful* mutants show a longer period of flower and fruit production due to prolonged meristem activity, which increases the number of flowers and fruits produced (Balanzà et al., 2018;

Martínez Fernández, 2017; Martínez-Fernández et al., 2024). As described before, *FUL1* and *FUL2* are required for fruit ripening in tomato and, together with *MBP20*, in controlling flowering time and inflorescence development. However, there are no reports of their putative function in the regulations of proliferative arrest.

In Chapter 1, we demonstrated that the control of sympodial and axillary meristem activation is critical to modulating the length of the reproductive period and, hence, the time to proliferative arrest. Based on *MBP20* specific expression pattern in early stages of reproductive development and in axillary meristems, and that mutants at *FUL1* and *FUL2* did not show phenotypes related to the duration of flowering, we selected *MBP20* to assess its role in proliferative arrest in tomato.

MBP20 gene was disrupted using CRISPR-Cas9 generating the *mbp20*^{del5} (-5 bp) and *mbp20*^{del10} (-10 bp) mutant alleles (Supplementary Fig 2.S.2A). Both mutants (obtained in the Micro-Tom cultivar) had deletions in the first exon of the *MBP20* gene, which likely resulted in truncated proteins affecting the function of the gene. Flowering time was evaluated as the number of leaves to the first inflorescence and the number of days to the first anthesis flower. Notably, both mutants showed a delayed flowering time, with a significant increase in the number of leaves to the first inflorescence compared to the wild type (Supplementary Fig S.2.2B-C). No obvious defects were observed in the development of leaves, flowers, or inflorescences (Fig 2.2).

We evaluated the role of *MBP20* in controlling the timing of proliferative arrest by monitoring flower and fruit production in the wild type and *mbp20* mutants in both determinate and indeterminate tomato backgrounds. In determinate tomato plants (*sp* background), *mbp20* mutants exhibited early proliferative arrest compared to the wild type (Fig 2.2B-C). Wild-type plants produced flowers for up to 7 weeks after the floral transition, while the mutants reached proliferative arrest by week ~5 (Fig 2B), observing a sharp decrease in flower production in the mutants in week 4 (Supplementary Fig. S.2.2D). This decline in the number of flowers per week was closely related to a reduction in the development of both primary sympodial units and axillary branches. The analysis of the sympodial units in the main shoot (Supplementary Fig. S.2.2I) showed that the mutants had fewer sympodial units, and these units were also shorter than those in the wild type (Supplementary Fig. S.2.2J). Similarly, axillary meristem activation was limited in the mutants, with fewer and shorter lateral branches (Supplementary Fig. S.2.2K and 2L).

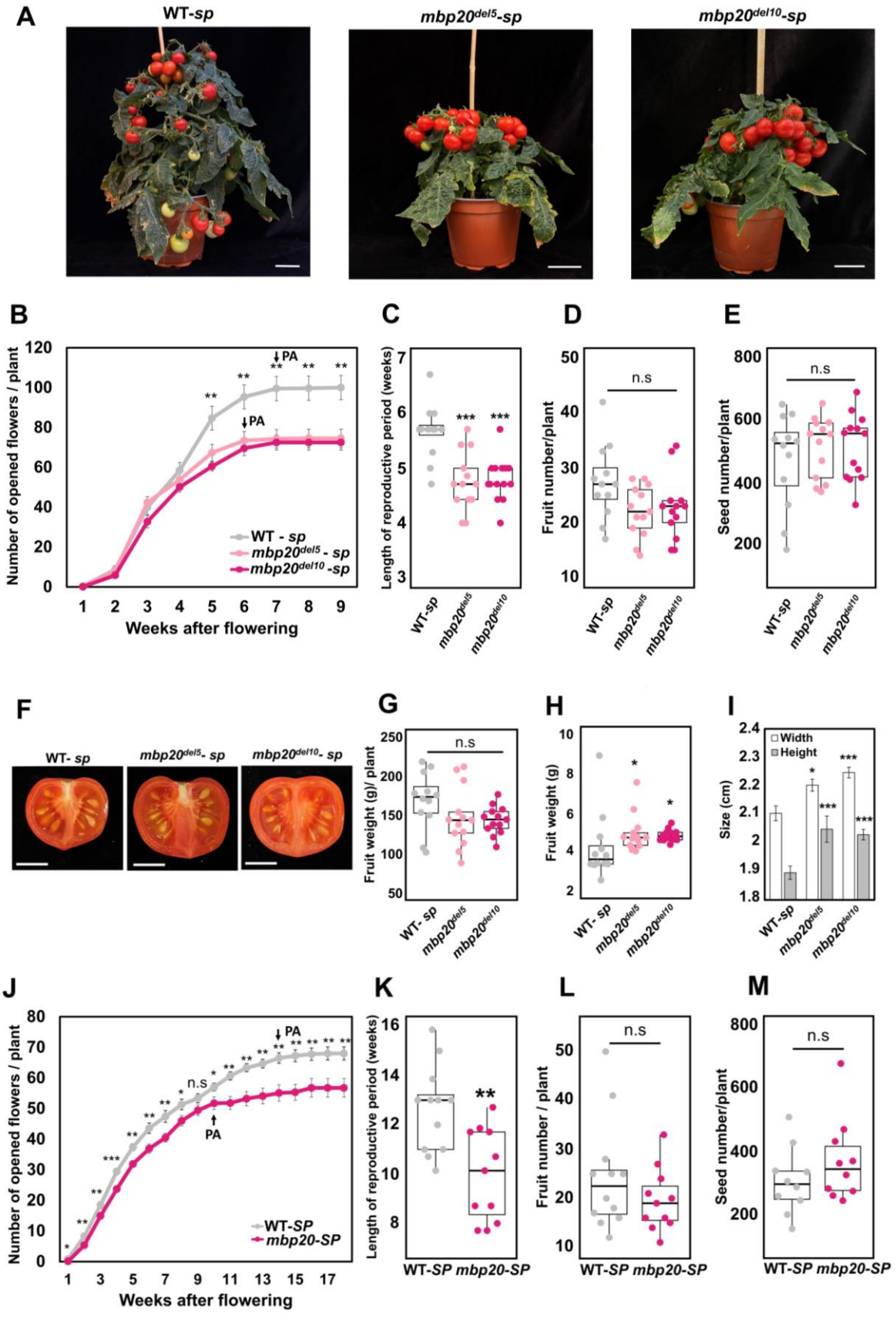

A
WT-sp
mbp20del5-sp
mbp20del10-sp
B
Number of opened flowers / plant
Weeks after flowering
PA
WT - sp
mbp20del5 - sp
mbp20del10 -sp
C
Length of reproductive period (weeks)
D
Fruit number/plant
n.s
E
Seed number/plant
n.s
F
WT- sp
mbp20del5- sp
mbp20del10- sp
G
Fruit weight (g)/ plant
n.s
H
Fruit weight (g)
I
Size (cm)
Width
Height
J
Number of opened flowers / plant
Weeks after flowering
n.s
PA
WT-SP
mbp20-SP
K
Length of reproductive period (weeks)
WT-SP mbp20-SP
L
Fruit number / plant
n.s
WT-SP mbp20-SP
M
Seed number/plant
n.s
WT-SP mbp20-SP

Figure 2.2. Functional characterization of *MBP20* during tomato proliferative phase. **A)** Plant phenotype at proliferative arrest of the wild type (*sp*), *mbp20*^{del5} and *mbp20*^{del10}. Scale bar indicated 5cm. **B)** Cumulative number of open flowers to proliferative arrest , **C)** Duration of the reproductive phase indicated in weeks from the first to the last flower opened **D)** number of fruits per plant and **E)** number of seeds per plant of indicated genotypes. **F)** Ripe fruit phenotype of wild type (*sp*), *mbp20*^{del5} and *mbp20*^{del10}. Scale bar indicates 1cm. **G)** Total fruit yield (g) per plant of each genotype. **H)** Fruit weight per genotype **I)** Quantification of the ripe fruit weight for each genotype **I)** Fruit size of wild type (*sp*), *mbp20*^{del5} and *mbp20*^{del10}**.** Data correspond to 160 fruits per genotype**. J)** Cumulative number of open flowers to proliferative arrest in WT (*SP*) and the *mbp20/SP* mutant. **K)** The length of the reproductive period in weeks from the first flower to the last flower opened from the main shoot. **L)** Total number of fruits produced per plant. **M)** Total number of seeds produced per plant. Significant differences were inferred according to the one-way ANOVA test followed by the HSD Tukey post hoc test in B, C, D, E, G and J; Kruskal-Wallis test in H, I and M; and the Student's t-test in K and L. Asterisks indicate significant differences: *$P < 0.05$; ***$P < 0.001$, n.s= no significant differences. Data correspond to n=11-13 independent plants. Error bars indicate the standard error of the mean (s.e.m).

In addition, we observed an increase in inflorescence branching in the mutants compared to the wild type, which predominantly displayed unbranched inflorescences (Supplementary Fig. S.2.2E and F). However, this branching did not result in a significant increase in the total number of flowers (Supplementary Fig. S.2.2G and Fig 2.2B). Thus, the limited activation of axillary meristems in the mutants had a more significant impact on the cessation of flowering than the increase in inflorescence branching.

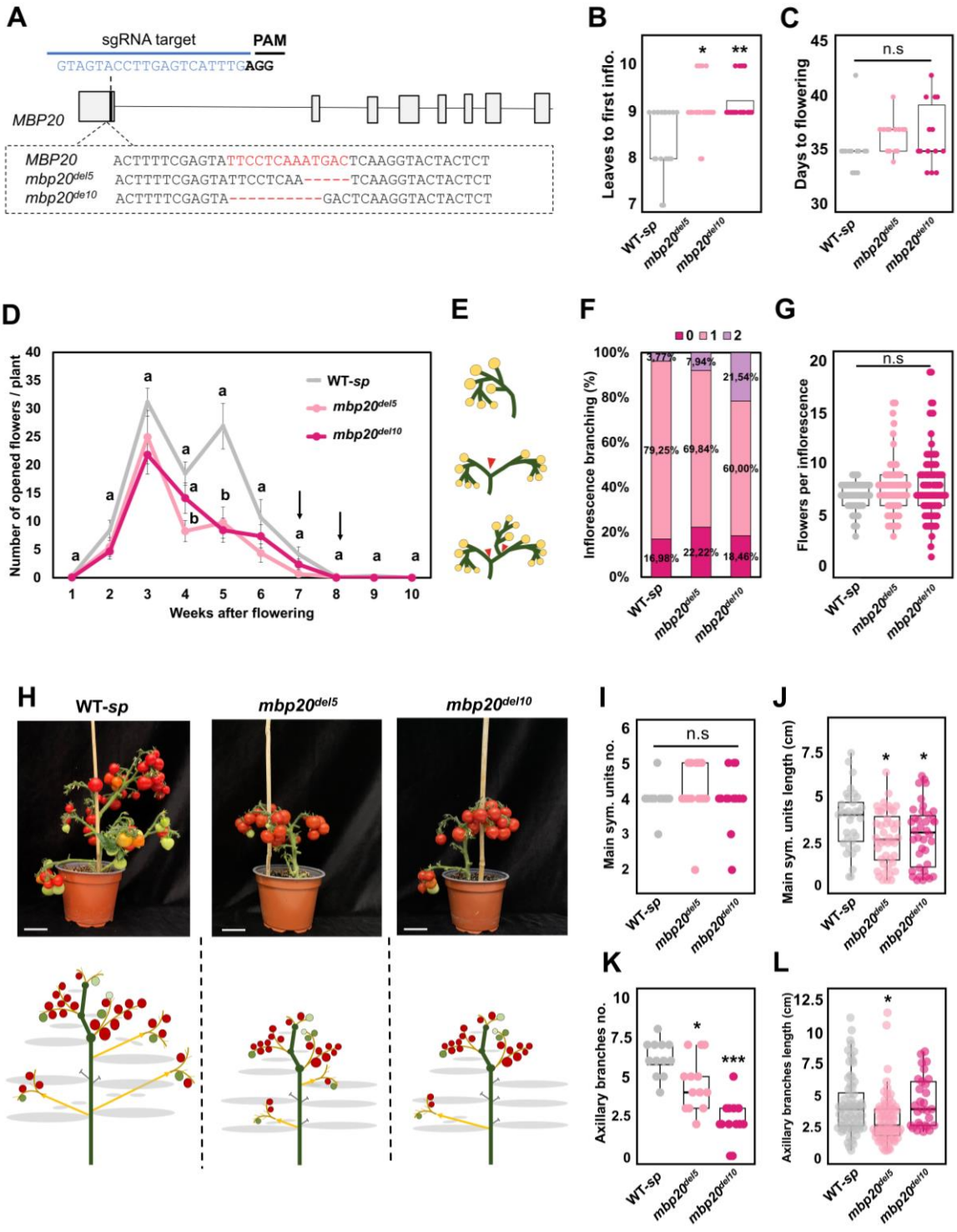


Figure 2.S.2. Phenotypical characterization of *mbp20* mutants with determinate growth (*sp* mutants).

A) A single guide RNA (in blue) was targeted to the first exon of the Solyc02g089210 (*MBP20*) gene. *mbp20* mutant sequences identified as *mbp20*^{del5} and *mbp20*^{del10} are highlighted in red on the box. **B)** Quantification of primary shoot flowering time in leaves to first inflorescence and **C)** days to first anthesis flower in wild-type (WT-*sp*) plants and *mbp20* mutants. **D)** Weekly flower production ratio of wild type (WT-*sp*), *mbp20* mutants from flowering initiation until the cessation

of flower production after proliferative arrest occurs (indicated by the black arrow). **E)** Diagram of tomato inflorescences in green with flowers represented by yellow circles showing 0, 1 and 2 branching events (indicated by red arrows). **F)** Proportion of inflorescence branching events (0, 1, 2) according to the indicated genotypes. **G)** Flowers per inflorescence developed by wild type (WT-*sp*) and *mbp20* mutants. **H)** Phenotype of the WT and *mbp20* plants at proliferative arrest. Leaves were removed to allow better visibility of the sympodial units and the developed lateral branches. The phenotype of these plants is illustrated below, where the leaves are shown in grey. Red and green circles show fruits at different stages of development. Yellow arrows represent lateral branches, and the leaf axils' grey lines indicate arrested axillary meristems. **I)** Sympodial units developed in the main shoot and **J)** their length in cm. **K)** Total **l**ateral branches developed and **L)** their length in cm.
Asterisks (**P < 0.05 ; **P < 0.01; ***P < 0.001,* n.s= no significant differences) indicate significant differences according to the Kruskal-Wallis test in B, C, D, I, the one-way ANOVA test followed by the HSD Tukey post hoc test in J, K and L. Different letters (a, b) indicate significant differences from each other. Data correspond to n=11-13 independent plants. Error bars indicate the standard error of the mean (s.e.m).

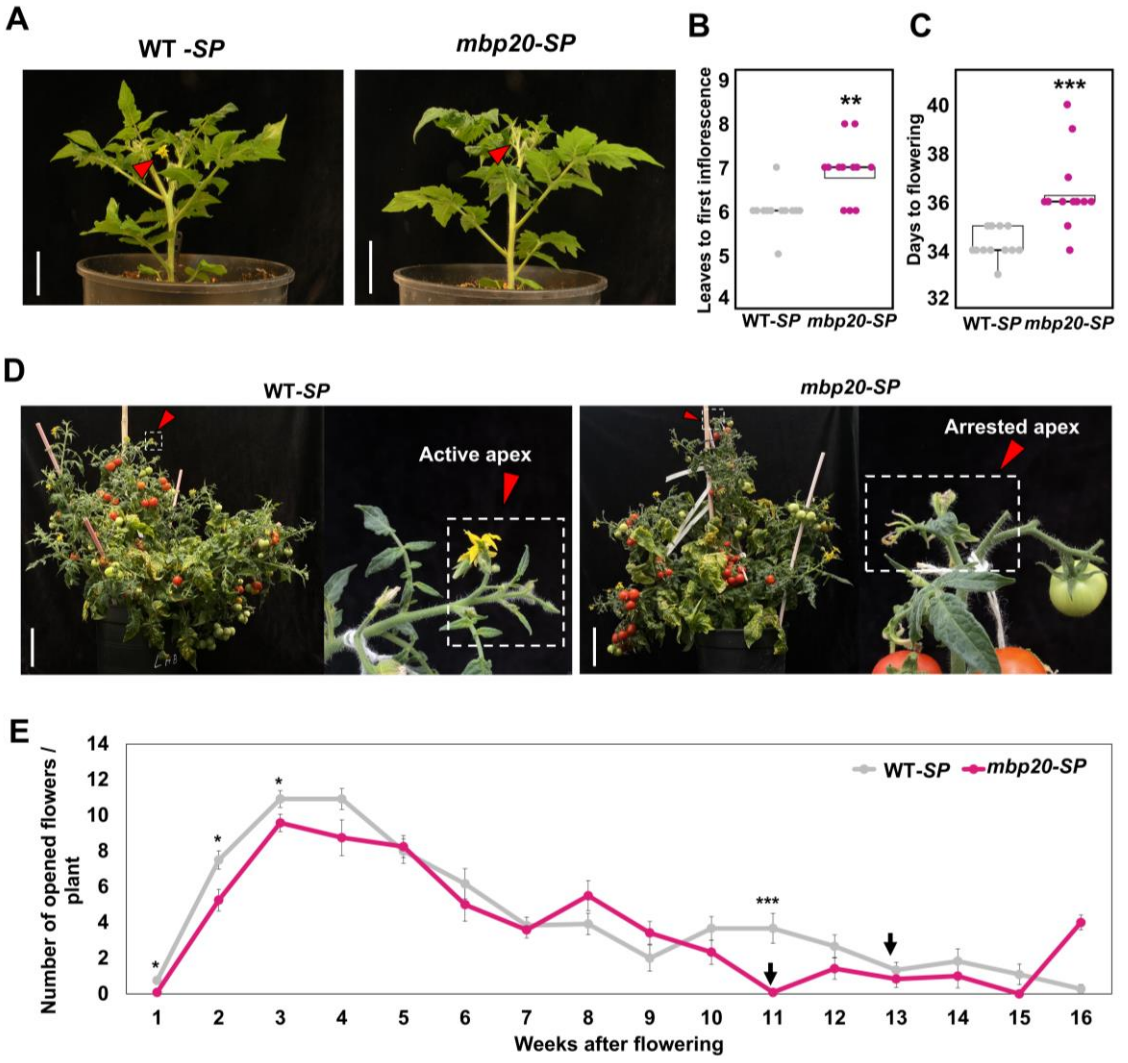
Interestingly, despite this early cessation of reproductive activity in *mbp20* mutants, the total number of fruits and the production of seeds were not altered (Fig 2D, 2E, and 2G). In addition, fruits from *mbp20* mutants showed a small but significant increase in fruit size and weight (Figs 2F-H). This increase in fruit size suggests that *MBP20* could play a role in resource allocation during fruit development, with the loss of *MBP20* resulting in larger fruits in a shorter reproductive period resulting in an early PA.

Previous studies suggest that *SP* could be regulated by *SlFULs* (Jiang, 2022; Jiang et al., 2022). To further this study and verify that the phenotype observed in *mbp20/sp* mutants was not due to the *sp* genetic background, we obtained *mbp20* mutants with an indeterminate growth habit by introducing a functional *SP* allele through crosses.

In the indeterminate background (*SP* gene restored), the loss of *MBP20* function had similar phenotypic effects to the ones shown in the determinate background. This included delayed flowering (Supplementary Fig 2.S.3 A-C), a reduction in sympodial unit number (Supplementary Fig 2.S.3 G), and early proliferative arrest (Fig 2.2J and K) with a decrease in flower production after week 11 (Supplementary Fig 2.S.3 E). Mutant plants (*mbp20-SP*) also produced an equivalent number of flowers per inflorescence (Supplementary Fig 2.S.3 H), fruits (Fig 2.2L), and seeds (Fig 2.2M) than the wild type (WT-*SP*).

Taken together, the results obtained in both determinate and indeterminate backgrounds, indicate that *mbp20* mutation causes early PA and that the reduction of sympodial

meristem activity is the primary cause of this early arrest. The results indicate that *MBP20* acts as a regulator of lifespan delaying meristem arrest in tomato plant. This function is the opposite to the described for *FUL* homologs in *Arabidopsis* and *Pisum sativum*, where mutants of these genes show a delayed proliferative arrest. *MBP20* has then an unexpected role in regulating the timing of proliferative arrest in tomato plants by controlling the development of sympodial units in a *SP*-independent manner.



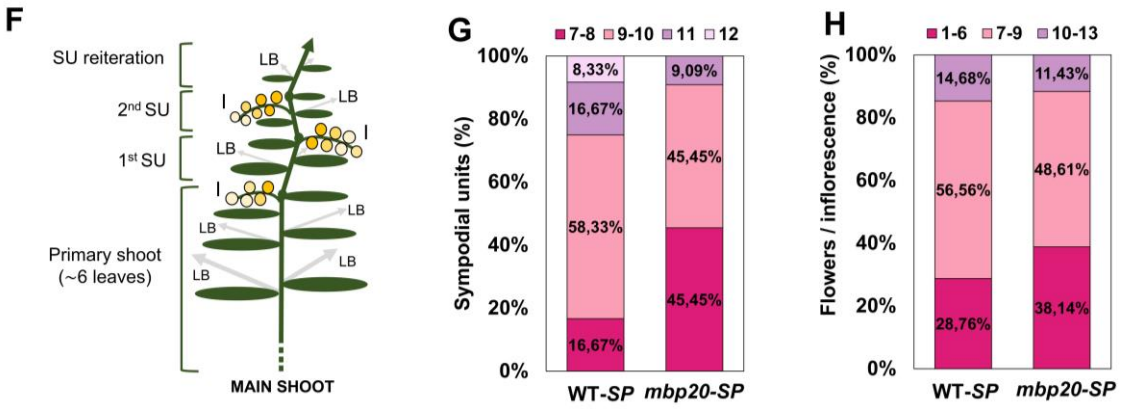


Figure 2.S.3. Characterization of *mbp20* mutant plants with indeterminate growth (*SP* restored) during reproductive development. A) Phenotype of wild type (WT-*SP*) and *mbp20-SP* at flowering initiation. The red arrow points to the floral apex of the primary shoot meristem. Scale bar represents 5 cm. **B)** Quantification of primary shoot flowering time in leaves to first inflorescence and **C)** days to first anthesis flower in wild type (WT-*SP*) plants and the mutant *mbp20-SP.* **D)** Phenotype of wild type (*SP*) at proliferative arrest and *mbp20-SP* mutant. The dotted white square marked by the red arrow indicates the active and arrested apex of the main shoot of each genotype. Scale indicated 10cm. **E)** Weekly flower production ratio of wild type (WT-*SP*) and *mbp2-SP* mutant from flowering initiation until the cessation of flower production after proliferative arrest occurs (indicated by the black arrow). **F)** Schematic diagram of an indeterminate wild-type tomato plant (WT-*SP*). The sympodial units (SU) of the main shoot and the leaves are represented in green. In the inflorescences (I), flowers at different stages of development are shown with yellow circles. Grey arrows in the leaf axils represent lateral branches (LB) and they are not included in the quantification of the parameters shown in this panel. **G)** Proportion of sympodial units developed and **H)** flowers per inflorescence for the indicated genotypes. Asterisks indicate significant differences according to the Mann-Whitney test in B, C, and E (**P < 0.05 ; **P < 0.01; ***P < 0.001*). Data correspond to n=11-12 independent plants.

miR172A regulates flowering time and meristem activity in tomato

Previous studies in *Arabidopsis* have shown that FUL and miR172 are negative regulators of *AP2* and *AP2-like* genes including *TOE1, TOE2, TOE3, SCHNARCHZAPFEN (SNZ),* and *SCHLAFMÜTZE (SMZ).* These *AP2-like* genes promote meristematic activity through *WUS* activation and modulation of cytokinin responses, regulating meristem activity throughout the reproductive cycle and, thus, the length of the inflorescence life span (Balanzá et al 2018, Martínez Fernández, 2017).

In *Arabidopsis thaliana*, five precursors of miR172 have been identified: *MIR172A*, *MIR172B*, *MIR172C*, *MIR172D*, and *MIR172E*. Similarly, four precursors have been found in tomato: *MIR172A*, *MIR172B*, *MIR172C*, and *MIR172D,* only missing *miR172E*. The mature miR172 sequence is highly conserved across species, consisting of a stable central backbone with variable nucleotides at the ends (Supplementary Fig.2.S.4 A)

In tomato, *AP2* genes are also targeted by miR172, and the *Solanum lycopersicum* genome contains 8 *AP2/AP2-like* genes (Sun et al., 2023), suggesting a large range of functions potentially due to gene duplication events (Supplementary Fig. 2.S.4 B). Phylogenetic analyses place *SlAP2a*, *SlAP2b*, and *SlAP2c* close to *Arabidopsis* AP2 and AtTOE3, while SlAP2d and SlAP2e, and specially SlTOE1 and SlTOE2 are more closely related to the *Arabidopsis* TOE1/2 genes, suggesting roles in flowering regulation and broader developmental processes such as organ identity (Supplementary Fig. 2.S.4 C). There are no tomato homologs to SMZ or SNZ, that appear to be specific to Brassicaceae.

Due to the elevated number of *SlAP2/AP2-like* in tomato and the likely functional redundancy among them, it is difficult to predict which homolog may have a more prominent role in PA control. For this reason, we proposed the overexpression of miR172 as a strategy to reduce the activity of the whole tomato *SlAP2/AP2-like* family and use these plants to analyze the role of AP2 genes in PA regulation.

All miR172s are presumed to regulate all *AP2-like* genes, but when they are considered individually, single mutants appear to affect specific processes (Lian et al., 2021; Ó'Maoiléidigh et al., 2021).

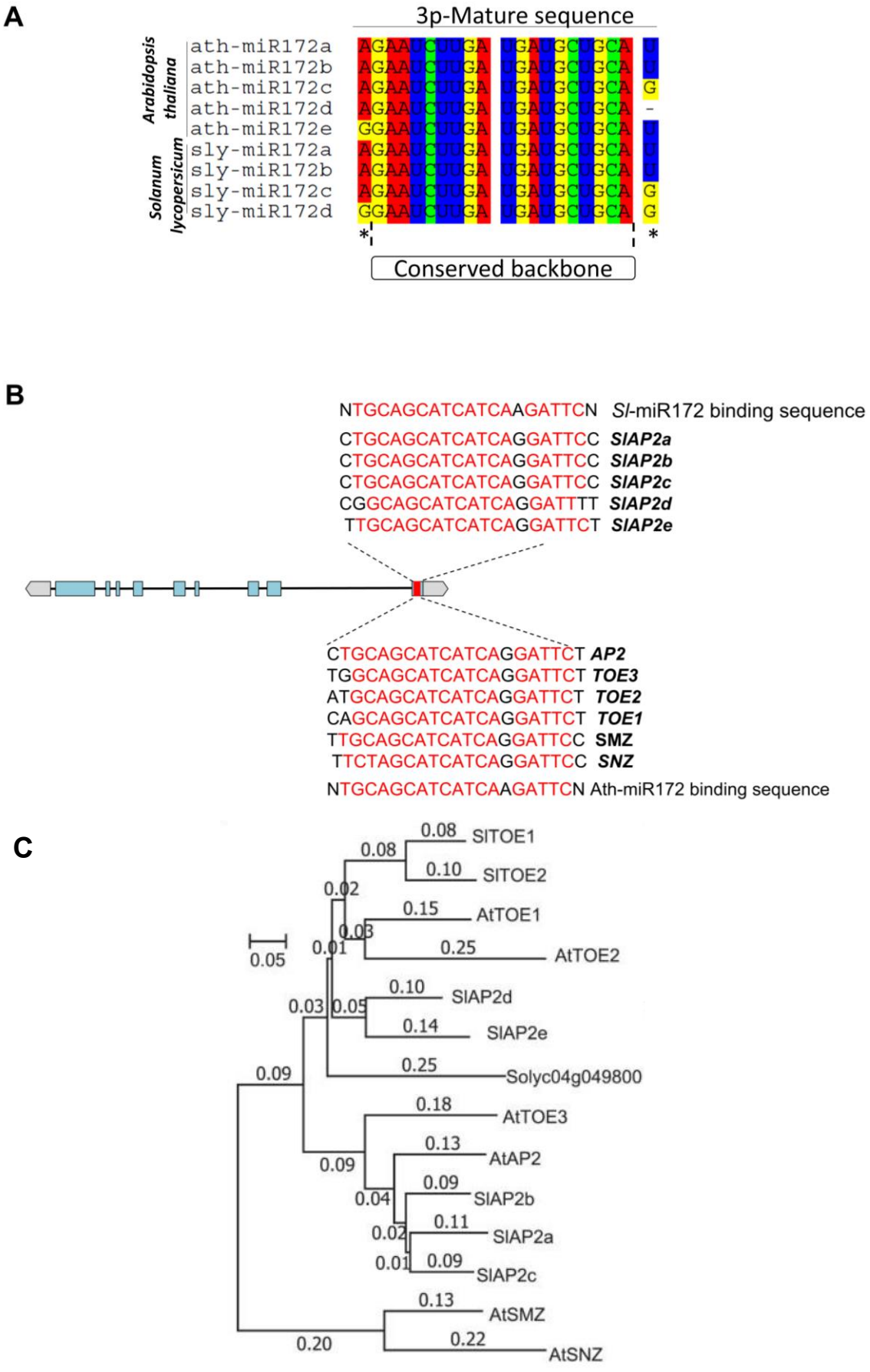
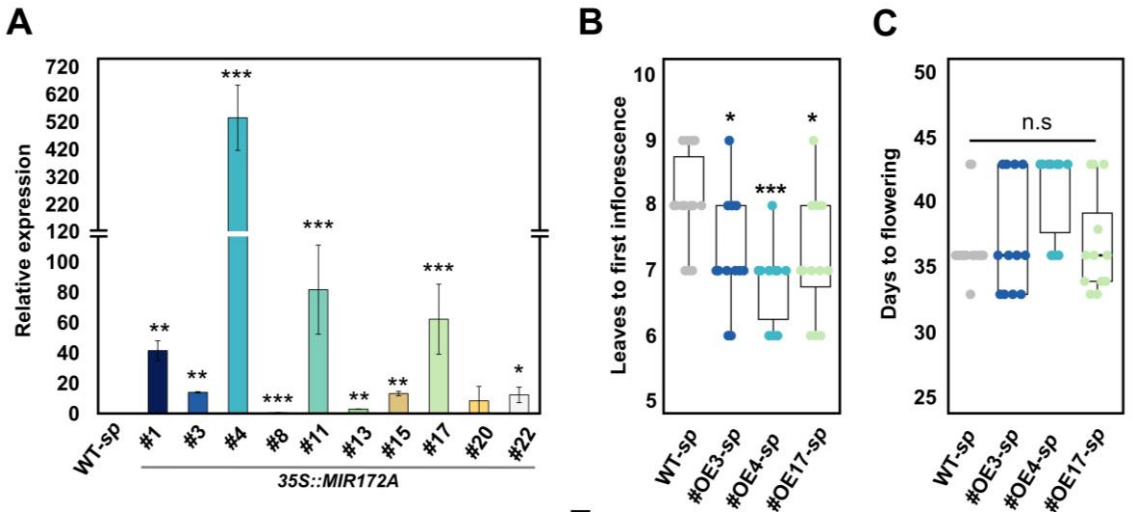

A
3p-Mature sequence
Arabidopsis thaliana
Solanum lycopersicum
ath-miR172a
ath-miR172b
ath-miR172c
ath-miR172d
ath-miR172e
sly-miR172a
sly-miR172b
sly-miR172c
sly-miR172d
Conserved backbone
B
NTGCAGCATCATCAAGATTCN Sl-miR172 binding sequence
CTGCAGCATCATCAGGATTCC SlAP2a
CTGCAGCATCATCAGGATTCC SlAP2b
CTGCAGCATCATCAGGATTCC SlAP2c
CGGCAGCATCATCAGGATTTT SlAP2d
TTGCAGCATCATCAGGATTCT SlAP2e
CTGCAGCATCATCAGGATTCT AP2
TGGCAGCATCATCAGGATTCT TOE3
ATGCAGCATCATCAGGATTCT TOE2
CAGCAGCATCATCAGGATTCT TOE1
TTGCAGCATCATCAGGATTCC SMZ
TTCTAGCATCATCAGGATTCC SNZ
NTGCAGCATCATCAAGATTCN Ath-miR172 binding sequence
C
0.05
SlTOE1
SlTOE2
AtTOE1
AtTOE2
SlAP2d
SlAP2e
Solyc04g049800
AtTOE3
AtAP2
SlAP2b
SlAP2a
SlAP2c
AtSMZ
AtSNZ

Figure 2.S.4. Analysis of sequence conservation of the AP2-miR172 module between *Arabidopsis thaliana* and *Solanum lycopersicum*. A) Comparison of miR172 precursors in *Arabidopsis thaliana* and *Solanum lycopersicum*. Five miR172 precursors (*MIR172A*, *MIR172B*, *MIR172C*, *MIR172D*, *MIR172E*) have been identified in *Arabidopsis*, while four precursors (*MIR172A*, *MIR172B*, *MIR172C*, *MIR172D*) have been identified in tomato. The mature miR172 sequence is highly conserved, consisting of a central backbone with variable nucleotides at the ends. **B)** Conserved nucleotide recognition sites of the mature miR172 in its AP2/AP2-like target sequences (highlighted in red), emphasizing the highly conserved nature of these binding sites across species. **C)** Phylogenetic tree of tomato AP2 proteins and selected homologs, illustrating the existence of eight *SlAP2/SlAP2-like* paralogs in tomato. Bootstrap values at each node were calculated from 1,000 trials. This phylogenetic tree is obtained from (Sun et al., 2023).



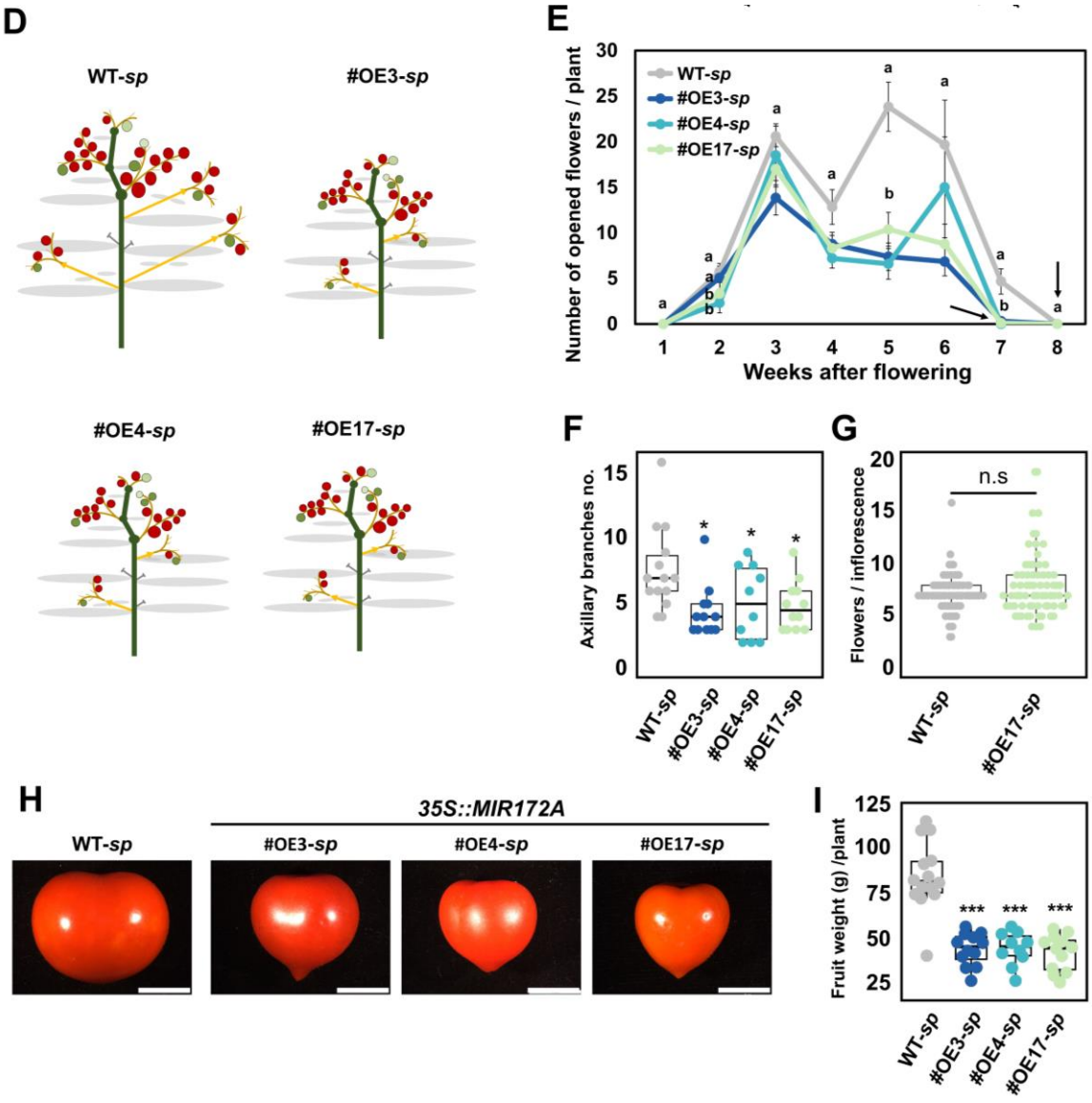


Figure 2.S.5. Effect of sly-miR172A overexpression in tomato plant architectures and fruit development. A) Relative transgene expression of the T_0 35S::MIR172A lines quantified by RT-qPCR in young leaves. The average data represented is three replicates ±SDs. SlActin8 was used as a reference gene. **B)** Quantification of primary shoot flowering time in leaves to first inflorescence and **C)** days to first anthesis flower in wild type (WT-sp) plants and the miR172A overexpression lines #OE3, #OE4 and #OE17.**D)** Diagram showing the phenotype of wild type (sp) plants and the miR172A overexpression lines #OE3, #OE4 and #OE17. Red and green circles show fruits at different stages of development. Yellow arrows represent lateral branches, and the leaf axils' grey lines indicate arrested axillary meristems. **E)** Weekly flower production ratio of wild type (sp) and miR172A overexpression lines from flowering initiation until the cessation of flower production after proliferative arrest occurs (indicated by the black arrow). **F)** Active lateral branches developing flowers and fruits in the genotypes indicated. **G)** Number of flowers per inflorescence of primary shoot. **H)** Fruit phenotype of wild-type (WT-sp) plants and miR172A overexpression lines (#OE3, #OE4, #OE17). Scale bar indicated 1cm. **I)** Fruit yield per plant. Data were analyzed with a Student's t-test in A, Kruskal-Wallis test in B, C, E, one-way

ANOVA test followed by the HSD Tukey post hoc test in F and I. Asterisks indicate significant differences: *P < 0.05; ***P < 0.001, n.s= no significant differences. Data sets marked with different letters (a, b) at the top indicate significant differences among groups. Data correspond to n=10-14 independent plants. Error bars indicate the standard error of the mean (s.e.m).

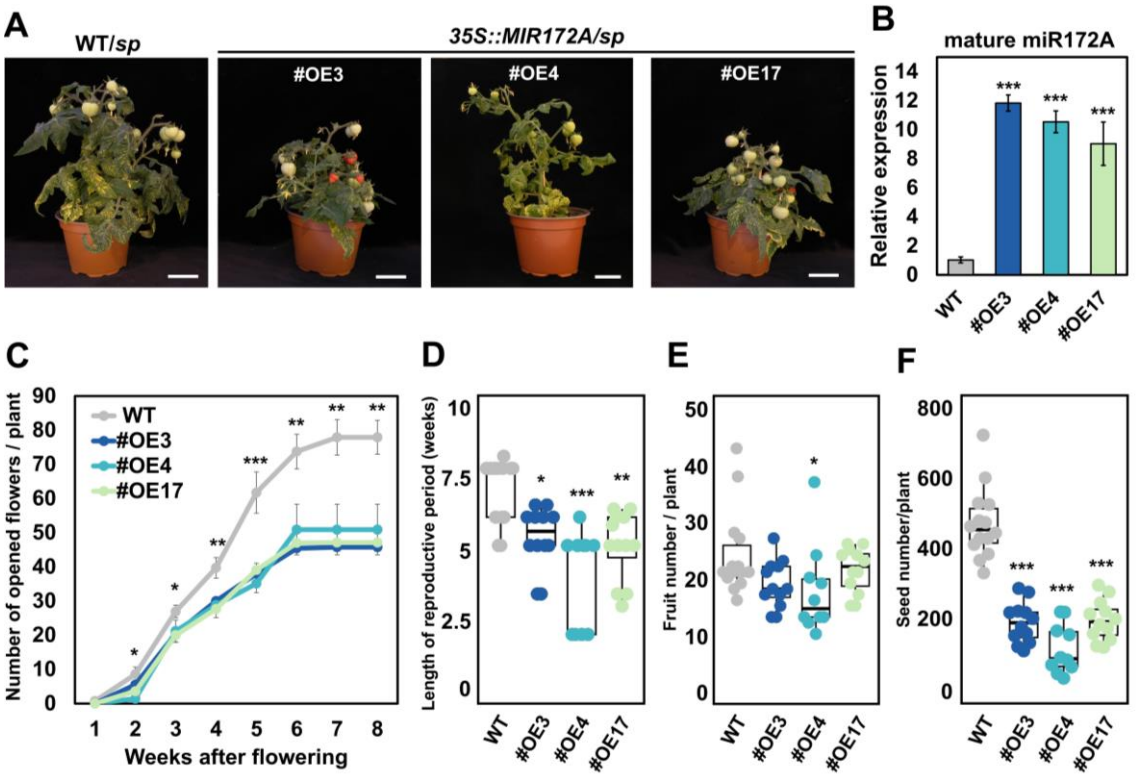
Ten independent miR172A overexpression lines (#OE-miR172) were generated in the determinate tomato Micro-Tom cultivar (MT-*sp*) that showed different expression levels of the transgene (Supplementary Fig. 2.S.5 A). We obtained homozygous plants for three of the lines (#OE3, #OE4 and #OE17) and quantified the levels of the mature miR172A. All of them showed similar and high levels of the mature miR172A compared to the wild type (WT-*sp*) (Fig 2.3B). The overexpression of miR172A resulted in a mild early flowering phenotype. Although the time to the first open flower was equivalent, the overexpression lines showed a reduction in the number of leaves of the main primary shoot (Supplementary Fig. 2.S.5 B-C). This data suggests that miR172A participates in the control of flowering time through the regulation of *SlAP2/AP2-like* genes, as occurs in *Arabidopsis* and *Pisum sativum* (Martinez-Fernandez 2017, Burillo E., 2024).

PA was evaluated in the overexpression lines by weekly monitoring flower production over time. The three independent lines exhibited earlier proliferative arrest and while wild-type plants produced flowers for up to 7-8 weeks before arresting, overexpression lines ended flowering around 5-6 weeks (Fig. 2.3A, C, D). In addition, we observed a reduction in the rate of weekly flower production in the plants overexpressing miR172A (Fig. 2.4E). Axillary meristem activity was also compromised in the overexpressing lines that showed a reduced lateral branch development compared to the wild type; fewer axillary meristems were activated in miR172A-overexpressing plants (Supplemental Fig. 2.S.5 D, F). In contrast, the number of flowers per inflorescence did not differ by genotype (Supplemental Fig. 2.S.5 G). These results suggest that high levels of miR172A accelerate the cessation of meristem activity and that is the cessation of axillary meristem activity what caused early proliferative arrest in #OE-miR172A lines.

Fruits and seeds have an important contribution to PA as source of signals that trigger the end of flowering. Fruits and seeds were recorded in plants overexpressing miRNA172 showing early PA. Despite the reduced number of flowers, the total number of fruits per plant did not significantly differ between miR172A overexpression lines and the wild type (Fig. 2.3E). This increase in the flower/fruit ratio observed in the #OE-miR172A lines suggests that fruit set is more efficient in the overexpressing lines. However, the overexpression of miR172 have a negative effect in fruit size and seed production (Fig. 2.3F). Fruits of the overexpression lines were significantly smaller

leading to a substantial reduction in total yield per plant (Supplemental Fig. 2.S.5 H and 5I).

Remarkably, despite the reduction in seed number in these lines, they showed an early proliferative arrest, suggesting that either these seeds were more active producers of the putative seed-to-meristem signal, or that overexpression of miR172 made the meristems more sensitive to such signal, although other hypothesis could be also proposed.



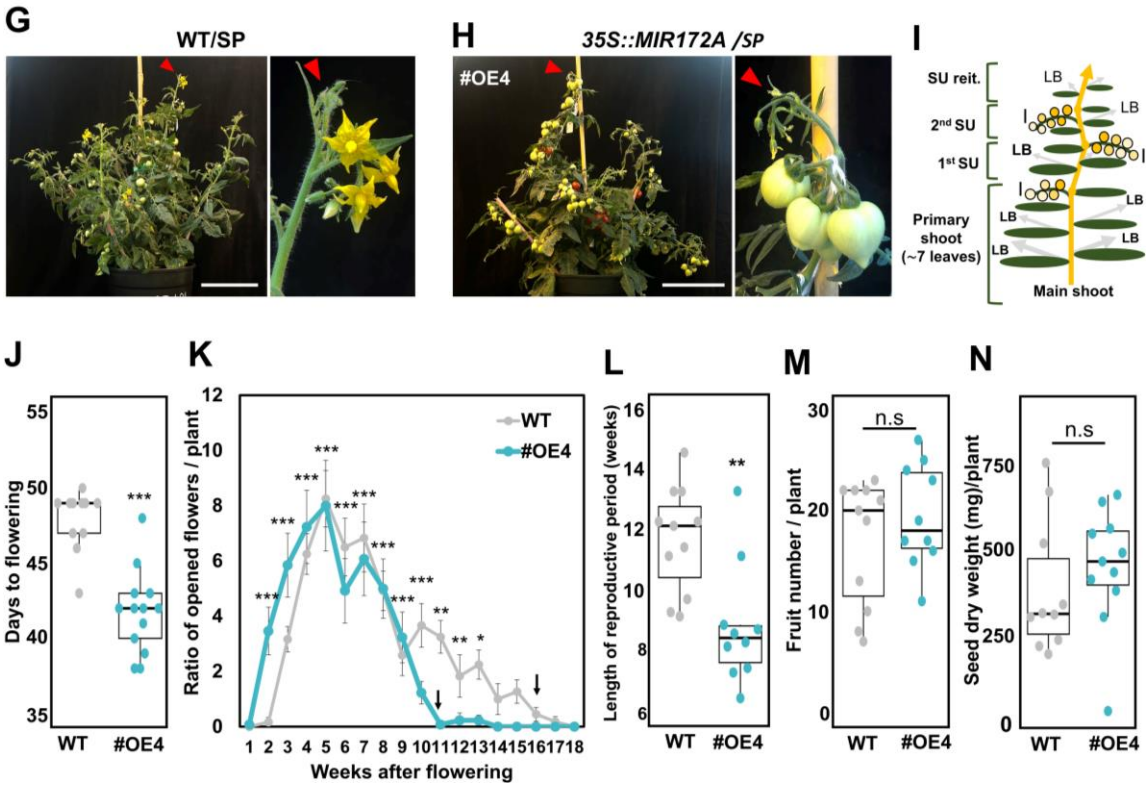


Figure 2.3. Functional characterization of miR172A during tomato reproductive phase. **A)** Phenotype of wild-type (WT-*sp*) plants and three miR172A overexpression lines (#OE3, #OE4, #OE17) at the time of proliferative arrest. Scale bar indicated 5cm. **B)** Relative expression by quantitative RT-PCR of mature miR172A in young leaves in wild type (WT-*sp*) and miR172A overexpression lines (#OE3, #OE4, #OE17). Data correspond to the average of three biological replicates ±SDs. *SlActin8* was used as a reference gene. **C)** Cumulative number of open flowers from flowering initiation to proliferative arrest. **D)** Duration of reproductive period indicated in weeks from the first to the last open flower **E)** number of fruits **F)** seeds per plant of indicated genotypes. **G-H)** Plant phenotype and main shoot apex at late stages of reproductive development. The red arrow points to the apex of wild-type (WT-*SP*) plants (active) and the miR172A overexpression line (arrested). **I)** Schematic representation of a wild-type indeterminate tomato plant (WT-SP) showing the main shoot with sympodial units (SU) and lateral branches (LB) in the leaf axils**. J)** Days to the first open flower in WT-*SP* and miR172A-*SP***. K)** Ratio of weekly flower production per plant from the beginning of flowering to the end of flowering in WT-*SP* and miR172A-*SP*. **L)** Length of time to proliferative arrest in weeks from flowering initiation to end of flowering. **M)** Total number of fruits and **N)** seeds dry weight (mg) per plant produced by the main axis.

Significant differences were inferred according to the Kruskal-Wallis test in C, D, and E, the ANOVA test followed by the HSD Tukey test in F, the Mann-Whitney test in J, K and N and the Student's t-test in B, M and L. Asterisks indicate significant differences: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, n.s= no significant differences. Data correspond to n=10-14 independent plants. Error bars indicate standard error of the mean (s.e.m).

We introgressed the transgene (*35S::MIR172A*) by crosses into the indeterminate Micro-Tom background (restored gen *SP*) to study the effect of miR172 and *SlAP2/AP2-like* genes in tomato plants with a different growth habit. We observed similar effects of miR172A overexpression. The *#OE4-SP* line showed early flowering, with a significant reduction in the number of days to first open flower compared to the wild type (WT-*SP*) (Fig. 2.3J). The timing of proliferative arrest was evaluated in the main axis of the plant (Fig. 2I). The overexpression line (#OE4-*SP)* also reached proliferative arrest earlier (Fig. 2.3G-H, L), with a complete cessation of flower production at week 11, 5 weeks before the wild-type (Fig. 2.3K), indicating an early decline in meristem activity. Again, the number of fruits produced on the main axis was not significantly different between both genotypes (#OE4-*SP* and WT-*SP*) (Fig. 2.3M), suggesting that, as in the determinate background, the fruit set rate in the overexpressing lines was higher. Surprisingly, we did not observe a reduction in the number of seeds produced per plant in the #OE4-SP line (Fig. 2.3N), which differs from the determinate cultivar (WT-*sp*), where there was a marked reduction in this parameter (Fig. 2.3N).

Taken together, these results indicate that miR172A overexpression promotes flowering termination by repressing the activity of axillary and sympodial meristems, highlighting miR172A as a key genetic factor in reproductive development and lifespan in tomato independent of the type of growth habit, determinate or indeterminate.

In Chapter 1 we have shown that seeds are a determining factor in the control of proliferative arrest. Determinate plants overexpressing miR172A, despite the drastic reduction in seed number, showed an early arrest, which made us wonder whether seeds could be overproducers of the signal, among other possible scenarios. To investigate the contribution of fruits and seeds in this early arrest, we performed a flower pruning experiment (removal of all flowers to prevent fruit set) on *35S::MIR172A* plants (Fig. 2.4A). In this experiment, we observed that *35S::MIR172A*-pruned plants did not reach proliferative arrest as the plant kept active indefinitely (Fig. 2.4B). Deflowering of *35S::MIR172A* plants to prevent fruit and seed formation caused the activation of axillary meristems, and the flower production rate increased considerably with respect to WT and to the untreated *35S::MIR172A* plants. However, despite continuous flower formation, we observed a sharp decrease in flower production rate in both WT and *35S::MIR172A* pruned plants at week 9, stronger in the latter (Figure 2.4C), suggesting a reduction in axillary meristem activity. Compared with unpruned controls, the flower production rate of these plants was still quite elevated, and from week 10 (when the experiment was terminated) the plants again showed an upward trend in the rate of

flowers produced. At this point, neither the WT-pruned nor the *35S::MIR172A*-pruned plants showed any sign of approaching the end of flowering (Figure 2.4C). Therefore although *35S::MIR172A-sp* overexpressing lines produce a reduced number of seeds, these are indispensable for coordinating early arrest along with miR172A.

In summary, while overexpression of miR172A reduces flower production and leads to early PA, deflowering experiments reveal that the seeds themselves are required for meristem arrest. These results indicate that miR172A may act in controlling proliferative arrest through a pathway that is at least partially independent of seed development, the most important factor triggering PA, according to our previous results (Chapter 1).

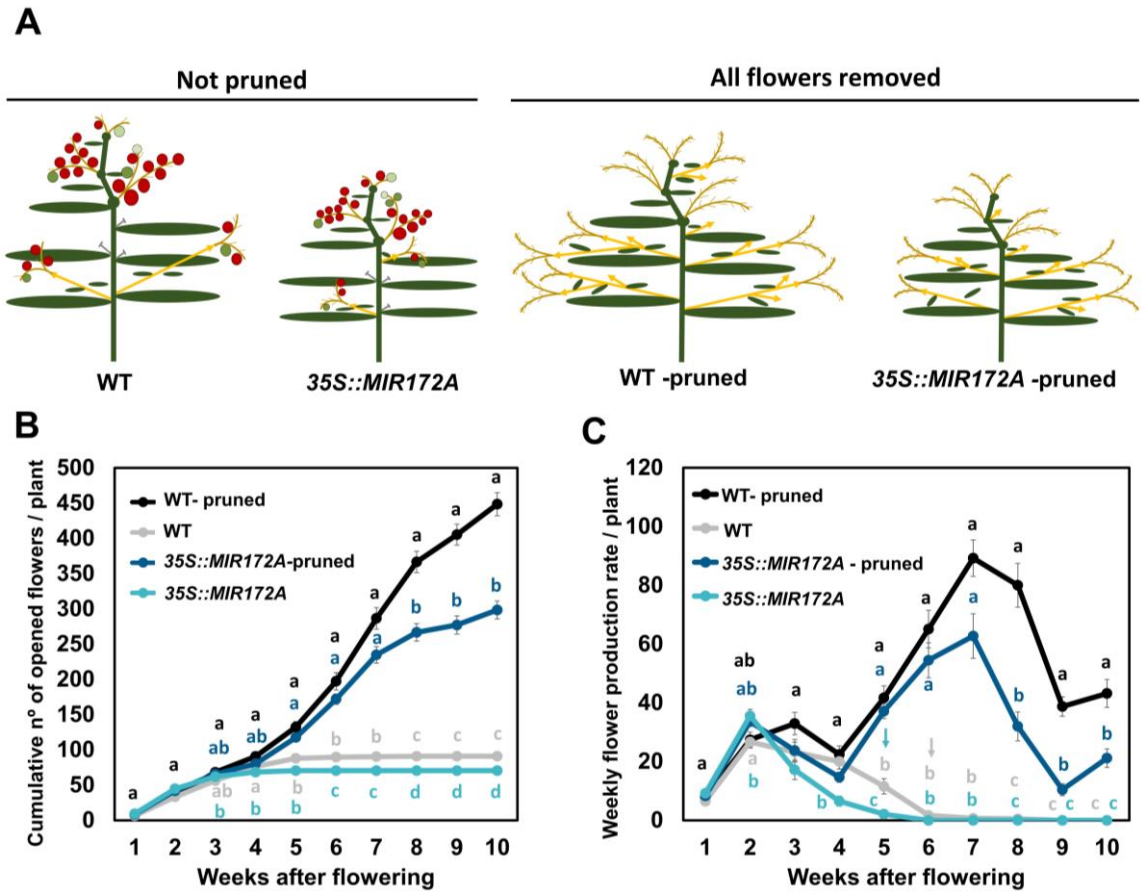


Figure 2.4. Effect of pruning on proliferative arrest of the miR172A overexpression line.
A) Diagram showing the phenotype of unpruned wild-type plants, unpruned miR172A overexpression line, and the indicated genotypes 10 weeks after flower pruning treatment preventing fruit set. Red and green circles show fruits at different stages of development. Lateral branches are represented by yellow arrows and the grey lines in the leaf axils indicate arrested axillary meristems. **B)** Cumulative number of flowers opened by the main and lateral sympodial units during 10 weeks of treatment after flowering. **C)** Flower production rate by main and lateral

sympodial units for 10 weeks of treatment after flowering. The black arrow indicates a relevant decrease in flower production of the unpruned plants: the wild type and the miR172A overexpression line. Significant differences were inferred according to the Kruskal-Wallis test. Data correspond to n=10-12 independent plants and different letters (a, b, c) indicate significant differences from each other. Error bars indicate standard error of the mean (s.e.m).

MIMICRY of miR172 Prevents Proliferative Arrest and Alters Tomato Fruit Development

As a complementary approach to the overexpression of miR172, we examined the effect of increasing *SlAP2/AP2*-like genes activity by inactivating endogenous miR172.

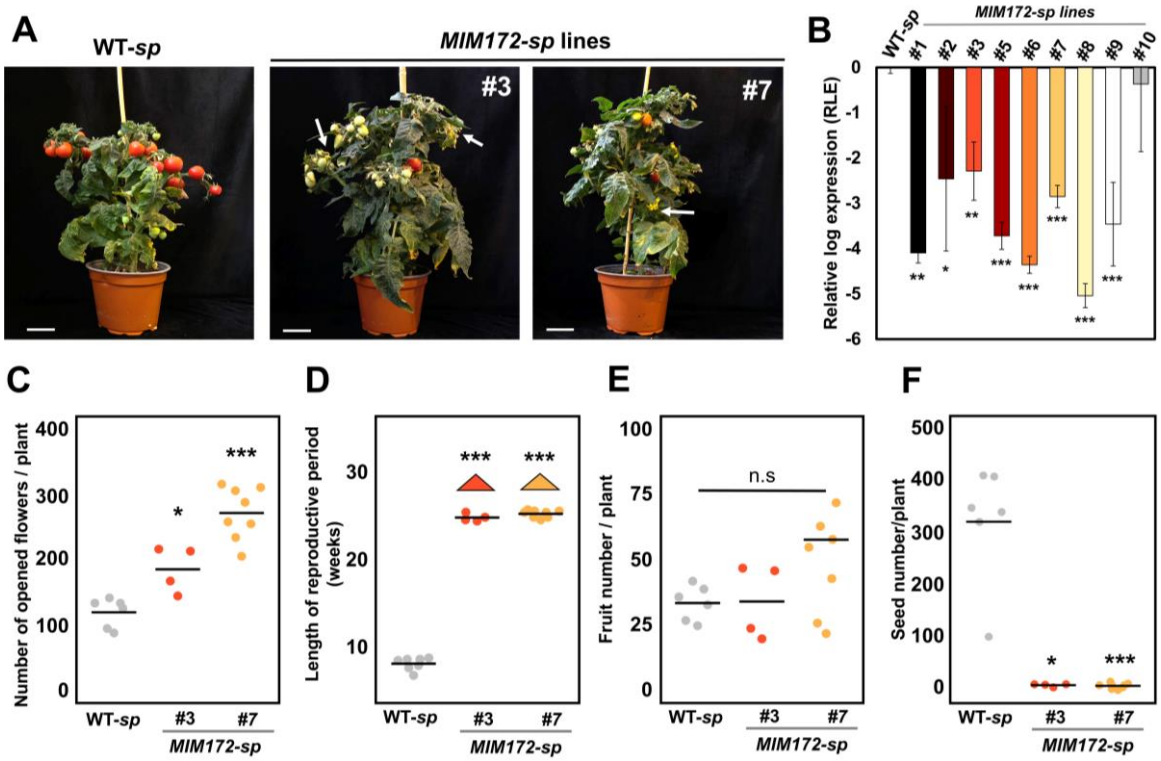
To specifically reduce the expression of miRNA172 in the plant, miR172 mimic lines (*MIM172*) were developed using the MIMICRY technology. In the MIMICRY method, an RNA sequence is designed to mimic the natural target site of the miRNA. By binding to the endogenous miRNA, it inhibits its interaction with its mRNA targets. This leads to the upregulation of genes normally repressed by the miRNA (Franco-Zorrilla et al., 2007). By blocking miR172 activity, the expression of its targets (specifically *SlAP2* and *SlAP2-like* genes) was expected to be enhanced, allowing us to investigate the role of *SlAP2/SlAP2-like* in controlling reproductive phase termination and meristem activity in tomato.

We evaluated the impact of miR172 suppression on flower production, the length of the reproductive period, and fruit development in both determinate (WT-*sp*) and indeterminate (WT-*SP*) tomato backgrounds (Fig. 2.5). In the determinate background (*sp* mutant), wild-type plants reached PA around 7-8 weeks after flowering, showing a clear cessation of flower production (Fig. 2.5A). In contrast, both *MIM172* lines (*MIM172* #3 and *MIM172* #7) exhibited a prolonged reproductive phase, with continuous flower production beyond the point of PA observed in the wild type. The expression analysis confirmed a significant reduction in mature miR172A levels in these lines (Fig. 2.5B), correlating with the prolonged reproductive activity observed (Fig. 2.5C-D). The number of flowers produced by the *MIM172* lines was significantly higher compared to the wild type, and the reproductive period extended for more than 25 weeks.

Despite the differences in flower production, the total number of fruits per plant was not significantly different between the wild type and MIM172 lines at the end of the experiment (25 weeks after flowering initiation) (Fig. 2.5E), although as the MIM172 lines were not arrested at this point, it could be expected that more fruits would be eventually produced. Regarding seed production, it was drastically reduced in the miR172 mimic lines (Fig. 2.5F), suggesting that miR172 suppression disrupts normal seed development or plant fertility.

In the indeterminate background (*SP* restored), the effect of miR172 suppression was also evident and equivalent to that observed in the *sp* background, with extended reproductive phase, many more flowers produced and a reduction in seed production (Fig. 2.5G-L)

In terms of plant architecture, both *MIM172-SP and MIM172-sp* lines showed a significant increase in the number of main sympodial units compared with wild-type plants, further indicating delayed PA (Supplementary Fig. 2.S.6 Q, Fig. 2.S.7 P). In addition, the total number of lateral branches was higher in the transgenic lines (Fig. 2.S.6 O,Q), suggesting that miR172 represses the activity of axillary meristem (AM) that contributes to the regulation of plant architecture and the timing to the end of flowering.



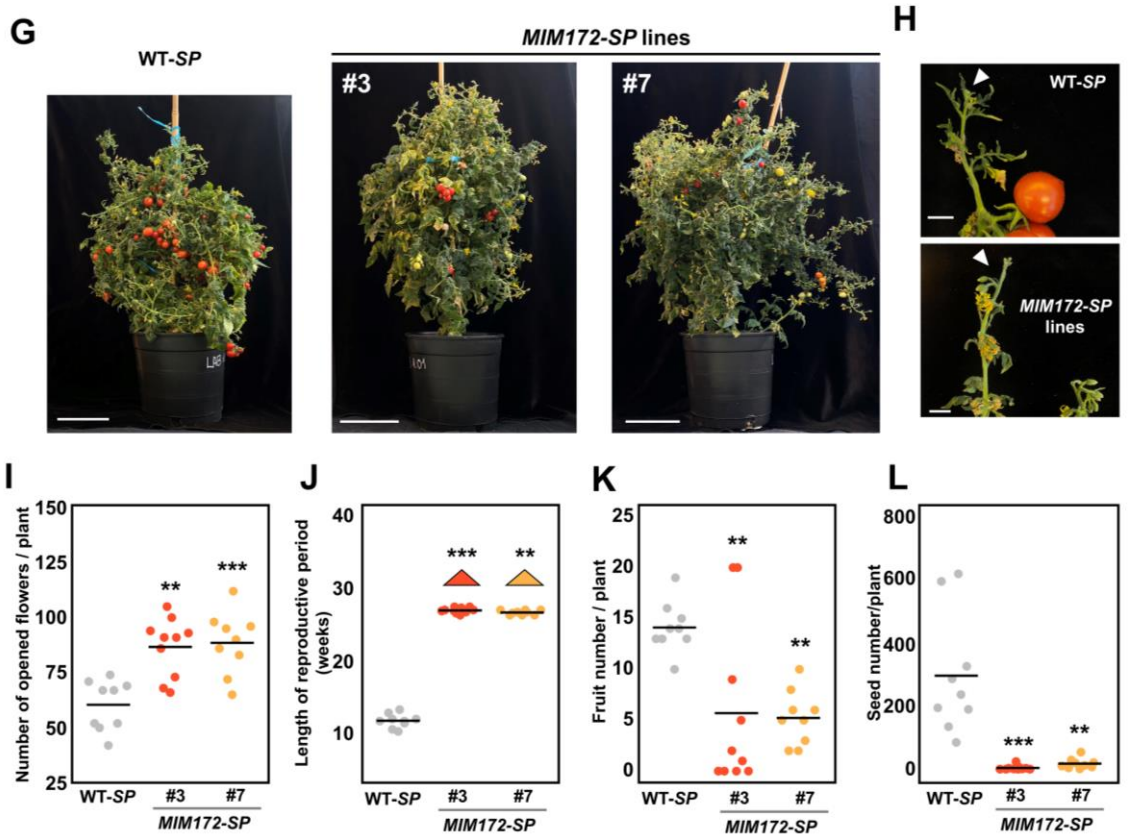


Figure 2.5. Analysis of *MIM172* lines during proliferative arrest with determinate (*sp* mutant) and indeterminate (*SP* restored) growth. A) Phenotype of determinate wild-type (WT-*sp*) plants and three *MIM172-sp* lines (*sp*#3 and *sp*#7) at late reproductive phase. Scale bar indicated 5cm. Wild-type plants (*sp*) are arrested while *MIM172-sp* lines show the development of new flowers (indicated by white arrows). Scale bar indicates 5 cm. **B)** Relative log expression (RLE) measured by quantitative RT-PCR of mature miR172A in young leaves of the wild type (WT-*sp*) and nine *MIM172* lines. Data correspond to T0 generation, the average of three replicates ±SDs is represented. *SlActin8* was used as a reference gene. **C)** Total number of flowers produced during the reproductive period of wild-type plants (WT-*sp*) and *MIM172-sp* lines evaluated 25 weeks after flowering. **D)** Length of reproductive phase in weeks from flowering initiation to the last flower open**.** The arrows in the graph indicate that *MIM172 sp*#3 and *sp*#7 lines remain active and did not undergo proliferative arrest. **E)** total number of fruits **F)** seeds per plant of indicated genotypes. Data correspond to n=4-7 independent plants. Error bars indicate the standard error of the mean (s.e.m). **G)** Phenotype of wild type (WT-*SP*) at proliferative arrest and *MIM172* lines (SP#3, SP#7). **H)** Representative apices of wild type (WT-*SP*) and *MIM172-SP* lines. The arrow points to the apex of wild-type (WT-*SP*) plants (arrested) and the *MIM172* line (active). **I)** Total number of flowers produced by the main shoot during the reproductive period of wild-type plants (WT-SP) until proliferative arrest occurs and *MIM172-SP* lines 25 weeks after flowering. **J)** Length of reproductive phase in weeks from flowering initiation to the end of flowering**.** The arrows in the graph indicate that *SP*#3 and *SP*#7 lines remain active and did not undergo proliferative arrest. **K)** Total number of fruits and **L)** seeds produced by the main axis per

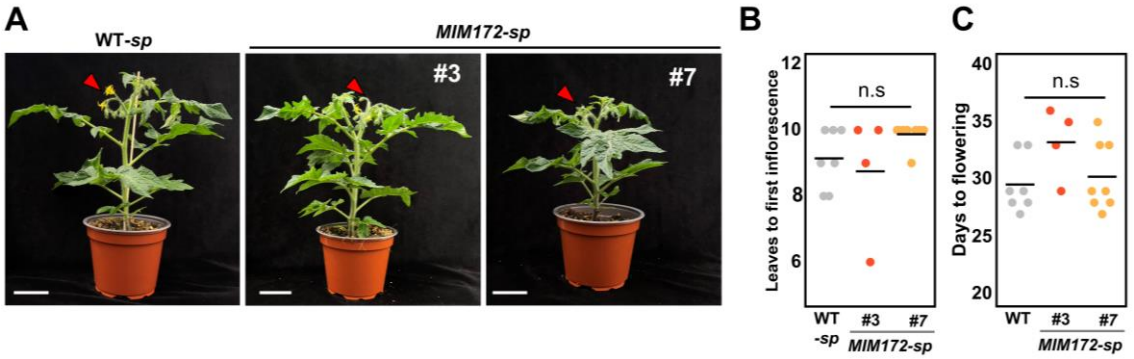
plant. Significant differences were inferred according to the Kruskal-Wallis test. Asterisks indicate significant differences: **P < 0.05; ***P < 0.001*, n.s= no significant differences. Data correspond to n=9-10 independent plants and error bars indicate standard error of the mean (s.e.m).

In addition to the prolonged extension in the reproductive period, some of the fruits of the *MIM172* lines showed morphological defects, with secondary fruits growing from the inside of the principal fruit (Supplementary Fig. 2.S.6 D-M; Fig. 2.S.7 D-M). This indeterminate fruit phenotype can be explained because miR172 sequestration prevents the repression of miR172 on *SlAP2/AP2-like* genes that promote *SlWUS* maintenance in the center of the floral meristem (Chu et al. 2019). (Karlova et al., 2011).

Despite these changes in fruit morphology, the overall fruit yield per plant was not significantly different between the genotypes (Supplementary Fig. 2.S.6 O) at the end of the experiment (25 weeks).

In summary, these results show that miR172 suppression by MIMICRY technology extends the reproductive phase and delays meristem arrest in both determinate and indeterminate tomato plants. In addition, they suggest that miR172 is involved in fruit set and in the repression of axillary and sympodial meristems, thus influencing both plant architecture and the transition to proliferative arrest.

All these results, together with those previously shown from the analysis of the *35S::miR172A* lines, reinforce that miR172, whose single targets are the *SlAP2/AP2-like* genes, is a key element in the control of meristem activity at the end of flowering.



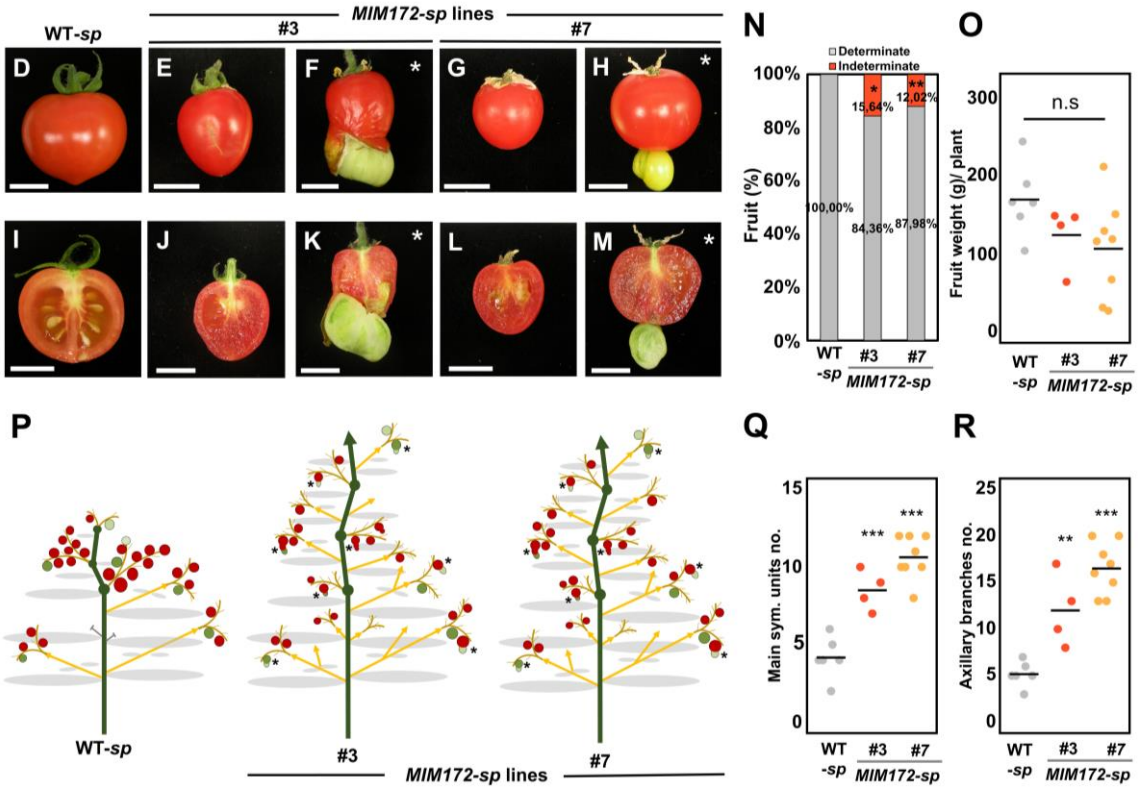


Figure 2.S.6. Effect of miR172A sequestration on flowering time, fruit development, and plant architecture in determinate (*sp* mutated) plants. A) Phenotype of wild type (WT-*sp*) and MIR172A lines at flowering initiation. The red arrow points to the floral apex of the primary shoot meristem. Scale bar represents 5 cm. **B)** Quantification of primary shoot flowering time in leaves to first inflorescence and **C)** days to first flower at anthesis in wild type (WT-*sp*) plants and the MIM172 lines (sp#3, sp#7). **D-M)** Phenotype of ripe fruits of wild type and lines sp#3 and sp#7. Panels marked with an asterisk (*) indicate fruit with an indeterminate phenotype. **N)** Proportion of determinate and indeterminate fruits developed in overexpression lines sp#3 and sp#7. **O)** Total fruit yield per plant in the indicated genotypes. **P)** Schematic diagram of indeterminate wild type plants (sp) and MIM172A lines (sp#3 and sp#7). Red and green circles show fruits at different stages of development. The main shoot is represented in green and the leaves in grey. Yellow arrows indicate lateral branches and the grey lines in the leaf axils represent arrested axillary meristems. (*) Asterisks indicate indeterminate fruit. **Q)** Number of sympodial units in the main shoot and **R)** Total lateral branches developed. Asterisks indicate significant differences according to the Kruskal-Wallis test in B and C, and the one-way ANOVA test followed by the HSD Tukey post hoc test in N, Ñ, P, and Q (*P < 0.05 ; **P < 0.01; ***P < 0.001, n.s= no significant differences). Data correspond to n=4-7 independent plants.

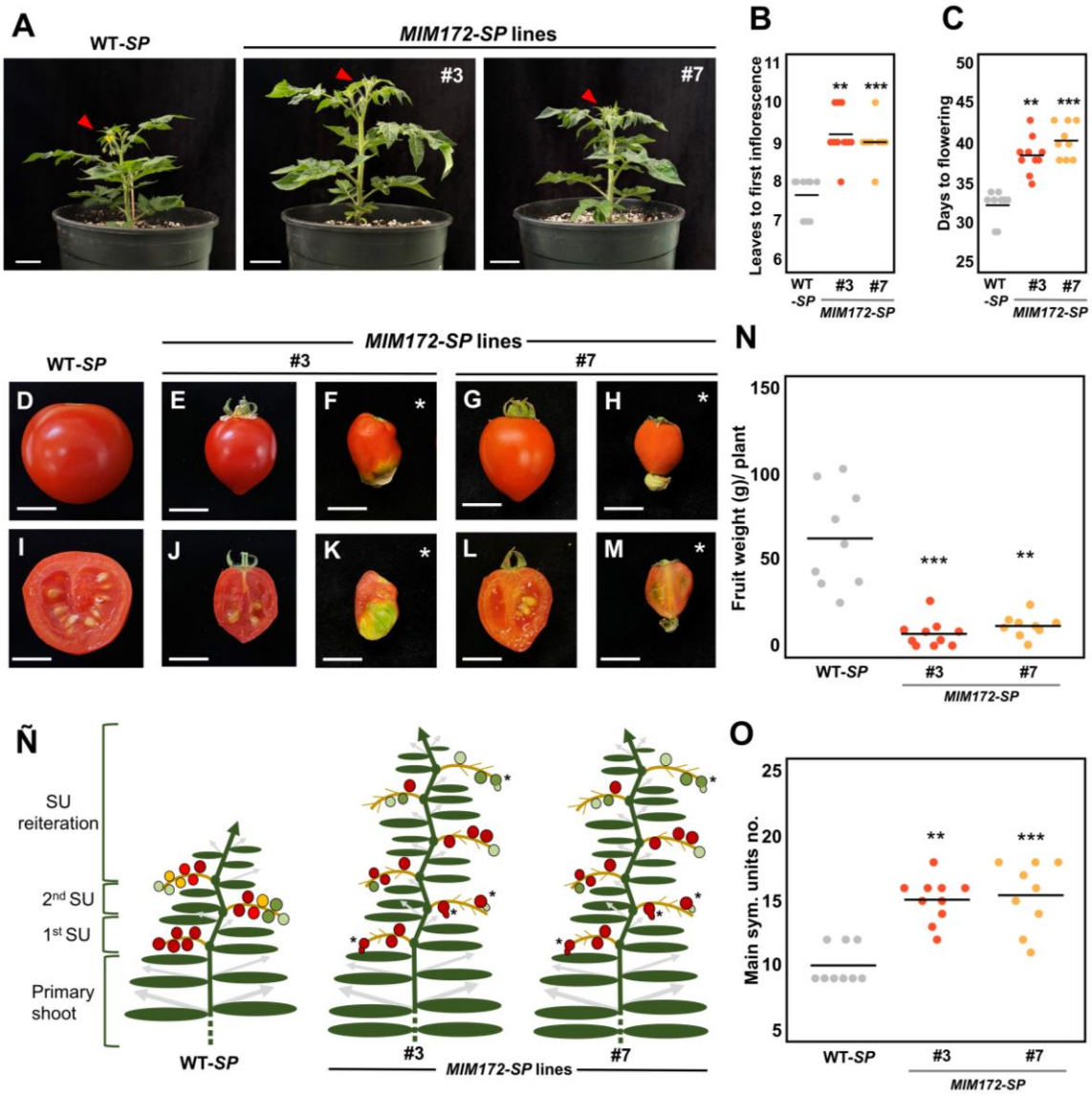


Figure 2.S.7. Effect of miR172A sequestration on flowering time, fruit development, and plant architecture in indeterminate (*SP* restored) plants. A) Phenotype of wild type (WT-*SP)* and *MIR172A* lines at flowering initiation. The red arrow points to the floral apex of the primary shoot meristem. Scale bar represents 5 cm. **B)** Quantification of primary shoot flowering time in leaves to first inflorescence and **C)** days to first flower at anthesis in wild type (WT-*SP)* plants and the *MIM172* lines (SP#3, SP#7). **D-M)** Phenotype of ripe fruits of wild type (WT-*SP)* and overexpression lines SP#3 and SP#7. Panels marked with an asterisk (*) indicate fruit with an indeterminate phenotype. **N)** Total fruit yield per plant in the indicated genotypes. **O)** Schematic diagram of indeterminate wild-type plants (*SP*) and *MIM172A* lines (SP#3 and SP#7). Red, orange, and green circles show fruits at different stages of development. (*) Asterisks indicate indeterminate fruits. The main shoot and leaves is represented in green. Lateral branches are illustrated in grey and are not considered in the panels plotted in this figure. **P)** Number of sympodial units in the main shoot and Asterisks indicate significant differences according to the Kruskal-Wallis test in B, C and N and one-way ANOVA test followed by the HSD Tukey post hoc test in P (***P < 0.01; ***P < 0.001)*. Data correspond to n=9-10 independent plants.

Discussion

Our results have uncovered important insights into the molecular mechanisms controlling proliferative arrest (PA) in *Solanum lycopersicum*, providing evidence for the functions of the miR172-SlAP2 module and *MBP20* at the end of flowering in tomato plants with determinate and indeterminate growth habit.

MBP20, an unexpected repressor of proliferative arrest

We selected *MBP20* as a candidate gene to participate in the regulation of proliferative arrest in tomato based on two premises. First, the specific expression pattern of *MBP20* during the early stages of reproductive development and in axillary meristems (Fig 2.1). Second, at the beginning of this work, only *FUL1* and *FUL2* had been functionally characterized and no phenotypes related to the end of flowering were described(Jiang et al., 2022),

Interestingly, the characterization of *mbp20* mutants showed that in the absence of *MBP20* function, the end of flowering is accelerated. This phenotype is unexpected when compared to the delayed PA shown by the mutants of the presumed FUL homologs in *Arabidopsis thaliana* and *Pisum sativum* (Balanzà et al., 2018; Martínez-Fernández et al., 2024). Actually, few mutants are known to cause early PA, particularly in *Arabidopsis* the fireworks (*fiw*) mutant, characterized as a gain-of-function allele of *CYSTEIN-RICH RECEPTOR-LIKE KINASE 14* (*CRK14*), has been shown to induce early proliferative arrest (PA) by suppressing *WUSCHEL* expression and acting downstream or independently of the AP2/FRUITFULL pathway (Imai et al., 2024), In addition other authors indicate that loss-of-function mutations within the AP2 family have also shown reduced reproductive period length (Burillo, 2024).

To understand our results, it is necessary to take into account that *MBP20* is closely related to *AGL79-like* genes. In *Arabidopsis*, the functional role of *AGL79-like* genes remains ambiguous. There are few reports on the characterization of *agl79* mutants, which show defects in root development, flowering time and apical dominance, although the mechanisms involved are not well described (Gao et al., 2018; H. Yang et al., 2023). In *Pisum sativum* and other legumes, *VEGETATIVE1 (VEG1/PsFULc)*, an *AGL79-like* gene, has a prominent role in inflorescence development, where it confers identity to the secondary inflorescence meristem, characteristic of the compound raceme of many legume species. (Berbel et al., 2012). Because *AGL79*-like genes have not been extensively characterized in other species, and their function in Arabidopsis and legumes

appears somewhat unrelated, the study of *MBP20* provides new knowledge to assess the functional evolution of the members of this clade.

The early proliferative arrest phenotype observed in *mbp20* mutants was similar in both determinate and indeterminate tomato plants. This indicates that the early proliferative arrest triggered by the loss of function of *MBP20* is independent of the *SP* (*SELF-PRUNING*) gene, which plays a crucial role in determining growth habit in tomato. This observation is interesting because, in *Pisum sativum*, genes analogous to *MBP20*, such as *VEG1*, are known to repress *DET* (a *TFL-like* gene)(Berbel et al., 2012) (Benlloch e al., 2015) If *MBP20* played a similar role in tomato, it would be expected that *SP* should be more active in the mutant background, which, based on the phenotypic characterization, did not appear to be the case. The fact that *mbp20* mutants show the same phenotype even in a *sp* background suggests that the role of *MBP20* in proliferative arrest is not mediated by the *SP* pathway, but rather points to its interaction with the *AP2* regulatory network.

One possible explanation for the observed phenotype is that *MBP20* may act not as a repressor of *AP2*, as traditionally assumed for *FUL* homologs, but rather as an activator. Alternatively, or even in parallel, *MBP20* might function antagonistically to other tomato *FUL* genes. This antagonistic interaction might lead to enhanced activity of other *FUL* genes (on *AP2* promoters or other targets) when *MBP20* is absent, thereby triggering the end of flowering. This hypothesis could be explored by quantifying *AP2* expression levels in *MBP20* mutants or by assessing whether *MBP20* directly influences the expression of downstream regulators of flowering also regulated by *FUL* genes.

Further studies would need to be conducted to test these hypotheses. To investigate the genetic network controlled by MBP20, DAP-seq or ChIP-seq assays could be performed to identify direct targets. On the other hand, analysis of high-order mutants may help to establish the function of individual *SlFUL* genes at the end of flowering and their functional interactions.

A conserved role of the miR172-SlAP2 module in controlling Proliferative Arrest in tomato

Our findings indicate that the miR172-SlAP2 module plays a conserved role in regulating the timing of proliferative arrest in tomato. As expected, overexpression of miR172 results in early proliferative arrest, while MIM172 lines exhibit delayed arrest, extending the flowering phase indefinitely. This supports the idea that miR172 is a key element in the control of the end of flowering by targeting genes of the *SlAP2/SlAP2-like* family, which is consistent with its conserved function across species (Martínez Fernández, 2017; Burillo, 2024).

However, an interesting observation emerged when considering the role of seed development. Despite producing fewer seeds, miR172-overexpressing lines still exhibited early arrest, raising the question of whether these lines are less sensitive to seed-derived signals. However, when fruit formation was prevented in these lines, the plants remained active for longer periods, indicating that miR172 alone is not sufficient to trigger the arrest.

One possible explanation is that miR172 may induce the production of additional signaling molecules, such as auxins or abscisic acid (ABA), within developing fruits or seeds, thereby amplifying the signal that leads to flowering termination. Future studies could investigate this by analyzing the exudates from miR172 fruit tissues to determine if they exhibit elevated levels of these hormones. Additionally, crossing miR172-overexpressing lines with seedless mutants (such as *slams*) could help clarify the role of fruit and seeds in these lines.

The results from the MIM172 lines further reinforce the role of miR172 in controlling the end of flowering. Inhibition of miR172 in these lines leads to an increase in flower and fruit production, indicating that miR172 indeed plays a significant role in regulating meristem activity in tomato. This is particularly revealing because while overexpression of miR172 reduces *AP2* levels, the effect seen in wild type backgrounds conditions may not rely on miR172. It is possible that other factors, such as *FUL* or additional regulatory genes, might also repress *AP2* in plant. However, the clear effect observed upon inhibiting miR172 in MIM172 lines provides stronger evidence for the miR172 essential role in this regulatory network.

Furthermore, the secondary fruit-like structures observed within the main fruit in MIM172 lines may be explained by the prolonged expression of *SlAP2/AP2*-like genes in developing ovaries, which could maintain *WUS* expression for an extended period. This sustained meristem activity could, in turn, result in indeterminate growth patterns, leading to the formation of fruit-inside-fruit phenotypes, similar to the mechanisms observed in misexpression of *miR156* and its impact on SPL/SBP transcription factors (Silva et al., 2014).

In summary, our data demonstrate that miR172 is a critical regulator of proliferative arrest in tomato, by modulating the levels and activity of AP2-like factors. In addition, the unexpected effect on the quantitative role of seeds from miR172 overexpressors, reveals a more complex scenario than previously understood. The interplay between miR172, *AP2*-like targets, and seed-derived signals reveals a finely tuned regulatory network that coordinates the end of flowering. Future research should focus on identifying the specific hormonal or genetic pathways influenced by miR172 and its targets, particularly through hormone assays and genetic crosses, to fully elucidate its role in this developmental process (Balanzà et al., 2018; Hensel et al., 1994; Litt & Irish, 2003; Sadka et al., 2023; Silva et al., 2014).

Biotechnological approaches to modulate PA in tomato

From a biotechnological perspective, modulating the timing of proliferative arrest (PA) in tomato offers significant potential for optimizing yield and crop efficiency. In this context, mutation of the *MBP20* gene emerges as a promising strategy. The *mbp20* mutants show early arrest of growth without compromising critical aspects of plant development, such as fruit or seed formation. In particular, these mutants produce larger fruits, which could directly translate into improved yield, both traits highly desirable in commercial agriculture. Breeding programs have focused on improving key traits such as fruit size, yield and disease resistance to meet agricultural demands. A remarkable case is the *fw2.2* locus, where mutations affecting the promoter sequence regulate cell division and drive the transition from small to large fruit during tomato domestication (Alpert et al., 1995; Frary et al., 2000). These improvements directly increase yield, making them highly desirable in commercial agriculture(Bai & Lindhout, 2007b).

In contrast, although overexpression of *miR172A* also causes early proliferative arrest, it presents considerable challenges. In some tomato varieties, overexpression of miR172A severely reduces fruit and seed yield, making it less suitable for practical

agricultural applications. Interestingly, this negative impact is not observed in indeterminate plants, where fruit and seed production remains constant on the main axis despite early PA. Studies at the whole-plant level taking into account fruits produced on lateral branches would be necessary to verify that fruit and seed yield is conserved in miR172-overexpressing plants in the indeterminate background.

By taking advantage of *MBP20* mutations, shorter growth cycles are achieved while maintaining or even increasing fruit size, thus improving crop management efficiency and productivity. This approach could be especially beneficial for breeding programs aimed at optimizing tomato varieties for faster turnover and higher yields.

Conclusion

In summary, our findings provide new insights into the genetic and molecular factors controlling proliferative arrest in tomato. While miR172 appears to have a conserved role in regulating meristem activity across species, *MBP20* may represent a case of neofunctionalization, where its loss leads to an early arrest phenotype. These results also highlight the complex interaction between genetic factors, resource allocation and seed-derived signals, providing a framework for future studies to manipulate flowering duration and yield in tomato and other crops.

Materials and Methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum L*.) seeds with determinate (Micro Tom sp/sp; MT-*sp*) and indeterminate (Micro Tom *SP/SP*; MT-*SP*) growth habits were used as control genotypes. Plants were grown in the greenhouse in 12 cm and 17 cm pots, respectively, with a peat:perlite mix (1:1 v/v) and irrigated with Hoagland's nutrient solution at 24/20 ºC (day/night) conditions. Natural light was supplemented with Osram lamps (150 µmol $^{m-2}$ $^{s-1}$, Powerstar HQI-BT, 400W) to obtain a 16h light/8h dark photoperiod when required.

Phylogenetic analysis

The phylogenetic tree was inferred using the Neighbor-Joining method (Saitou & Nei, 1987)(Felsenstein, 1985). The data set included 15 *FUL-like* genes from different plant species such as *Solanum lycopersicum*, *Arabidopsis thaliana*, *Pisum sativum*, and *Petunia hybrida*, obtained from the GenBank, TAIR, and Phytozome databases. The tree was rooted using the Arabidopsis *SEPALATA* and *AGAMOUS-LIKE* genes as outgroup. MEGA11 software was used to perform the evolutionary analysis (Tamura et al., 2021).The accession numbers are detailed in Table 2.S1.

Table 2.S1- Accession numbers of gene sequences from different plant species used in the phylogenetic analysis

Name	Species	Gene ID
PsFULa	*Pisum sativum*	*AY884287*
PsFULb	*Pisum sativum*	*AFI08227*
PsFULc	*Pisum sativum*	*AFI08225*
PsAP1	*Pisum sativum*	*AAL66379*
SlFUL1	*Solanum lycopersicum*	*Solyc06g069430*
SlFUL2	*Solanum lycopersicum*	*Solyc03g114830*
SlMBP10	*Solanum lycopersicum*	*Solyc02g065730*
SlMBP20	*Solanum lycopersicum*	*Solyc02g089210*
SlMC	*Solanum lycopersicum*	*Solyc05g056620*
AtFUL	*Solanum lycopersicum*	*At5g60910*
AtCAL	*Arabidopsis thaliana*	*At1g26310*
AtAP1	*Arabidopsis thaliana*	*At1g69120*
AtAGL79	*Arabidopsis thaliana*	*At3g30260*

AtSEP1	*Arabidopsis thaliana*	*At5g15800*
AtSEP2	*Arabidopsis thaliana*	*At3g02310*
AtSEP3	*Arabidopsis thaliana*	*At1g24260*
AtSEP4	*Arabidopsis thaliana*	*At2g03710*
PhFBP29	*Petunia hybrida*	AF335245
PhFBP26	*Petunia hybrida*	AF176783
PhAP1	*Petunia hybrida*	MK598839
PhPFG	*Petunia hybrida*	AF176782

RNA *in situ* hybridization in tomato meristem.

Meristem apices of plants 6, 10, and 13 days after germination (DAG) samples were collected and fixed in FAE (4% formaldehyde, 5% acetic acid, 50% ethanol) overnight at 4°C and then replaced with 70% ethanol. Following this, the samples were preserved in 70% ethanol at 4°C. The samples were embedded in paraffin in an automatic tissue processor (Leica TP1020).

To design the riboprobes, we selected two specific fragments from the 5' and 3' region sequence of each tomato *FUL*-homologs. cDNA fragments of 372, 427, and 461 bp were used for *FUL1*, *FUL2*, and *MBP20*, respectively. These fragments were cloned into the pGEM-T Easy vector (Promega). The RNA antisense and sense probes were generated using the T7 and SP6 polymerases from the cloning vector. RNA *in situ* hybridization with digoxigenin-labelled probes was performed as described previously (Gómez-Mena & Roque, 2018).

Primers used to generate the fragments are listed in Table 2.S2.

Plasmid assembly

Design of gRNA and CRISPR/Cas9 construct for MBP20 gene editing

A single gRNA with the highest score and the lowest number of off-target genes was selected to target the *MBP20* gene using the CRISPR-P v2.0 online tool (Liu et al., 2017). (Lei et al., 2014) We assembled the CRISPR/Cas9 construct with the GoldenBraid (GB) modular toolkit (Vazquez-Vilar et al., 2016). First, we used the "GB-CRISPR domesticator" tool to adapt the 20nt target sequence according to the GB system. Next, we combined the adapted target sequence with the scaffold RNA (GB0645) and U626 promoter (GB1001) into the destination vector pDGB3α1 to obtain the guide RNA expression cassette. In successive GB reactions, this cassette was assembled with

hCas9 (GB0639) and *nptII* (GB1181) transcriptional units into a destination vector. The final CRISPR/Cas9 construct was verified by restriction enzyme digestion and subsequent sequencing. *Agrobacterium tumefaciens* strain LBA4404 was transformed with the CRISPR/Cas9 construct to then proceed to plant transformation.

Design of miR172 mimic lines (MIM172) and miR172A overexpression constructs

To obtain the *35S:MIM172* construct, the MIM172 fragment was obtained from pCR8-MMI172 plasmid (Tesis Martínez Fernández, 2017). The pCR8-MIM172 plasmid was digested with *PvuII* and two fragments of 2.2kb and 1.2kb were obtained. The 1.2kb fragment containing the MIM172 fragment was purified and then used for assembling into the binary destination vector pK2GW7 that contains a 35S promoter and *NPTII* gene using Gateway recombination technology (Karimi et al., 2002).

To generate the 35S:miR172 construct the mature miR172A was expressed using the two-step MIR2390 method (Carbonell et al., 2014). Using the P-SAMS web tool (Fahlgren et al., 2016), the miR172A was selected and assembled into the miR390 backbone. This fragment was cloned into a pENTR™/D-TOPO® and subsequently into the pK2GW7 vector containing the 35S promoter and the *NPTII* selection gene using Gateway recombination technology (Karimi et al., 2002). The primers used are detailed in Table 2.S2.
The vectors containing the final constructs (Fig. 2.A1) were verified by enzyme digestion and sequencing, and then introduced into *Agrobacterium tumefaciens* strain LBA4404 to be used in the transformation of tomato plants.

Tomato plant transformation and genotyping of transformed plants.

We obtained transformant plants according to the method described by (Ellul et al., 2003). *Agrobacterium tumefaciens* strain LBA4404 containing the construct of interest, was co-cultured with axenic explants of tomato cotyledons. Regenerated tomato plants were selected and rooted in the presence of kanamycin (100 mgL^{-1}). The diploid plants were identified by flow cytometry and acclimated in the greenhouse for further analysis.

Genomic DNA from putative transgenic tomato plants was extracted and used in PCR reactions to amplify the corresponding transgene. The type and efficiency of edition of the *MBP20* gene was analyzed by amplifying a 651 bp fragment flanking the targeted sequence using the MBP20 For/Rev primers. The amplified fragments were purified and sequenced to analyze the presence and type of mutations using the online tools Tracking

of Indels by Decomposition (http://shinyapps.datacurators.nl/tide/) (Brinkman et al., 2014) and ICE 2 CRISPR (https://ice.synthego.com)..

Genotyping of the 35S:MIM172 and 35S:miR172A overexpression lines were conducted by amplifying a 656 bp and 384 bp fragment with the primers pairs 35S For/MIM172 Rev and miR172A For/miRT35S Rev, respectively.

The primers used are detailed in Table 2.S2 of the supplementary material.

Expression analyses by qRT-PCR.

To quantify mature miRNas, total RNA, including miRNas, was extracted from frozen leaves using the miRNeasy Micro Kit (QIAGEN) and treated with DNaseI (ThermoFisher Scientific) to remove genomic DNA. RNA purity and concentration were verified on a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific). RT-PCR was performed using the stem–loop quantitative reverse transcription PCR method (Varkonyi-Gasic et al., 2007). First-strand cDNA synthesis was synthesized from 1 µg of DNase-treated RNA using SuperScript IV reverse transcriptase (Invitrogen) using oligo dT or stem-loop RT primers listed in Table 2.S2. qRT-PCR reactions were run on a QuantStudio 3 (Thermo Fisher) using the MasterMix qPCR ROX PyroTaq EvaGreen.

Relative expression levels were calculated using the ΔΔCT method (Livak & Schmittgen, 2001). Results were normalized to the expression of the reference genes *SlActin8/CAC*. The sequences of the primers used showed an optimal efficiency value and are listed in Table 2.S2.

Table 2.S2- Primers used in this study.

Oligo name	Sequence (5'->3')	Vector/Gene ID	Experiment
MBP20 CRP For	ATTGTAGTACCTTGAGTCATTTG	Solyc02g089210	CRISPR guide *MBP20*
MBP20 CRP Rev	AAACCAAATGACTCAAGGTACTA		
SlMBP20 For	ATGGGAAGAGGTAGGGTAGAG	Solyc02g089210	*MBP20* edition analysis
SlMBP20 Rev	AAACGGGAGCTAAGAAGTATGAA		
mir172A_390 For	TGTAAGAATCTTGATGATGCTGC ATATGATGATCACATTCGTTATCTA TTTTTTATGCAGCATCCTCAAGAT TCT	-	35S:miR172 construct

mir172a_390 Rev	AATGAGAATCTTGAGGATGCTGCATAAAAAATAGATAACGAATGTGATCATCATATGCAGCATCATCAAGATTCT	-	
35S For	TTTCATTTGGAGAGGACTCCGG	pK2GW7	Genotyping of 35S:MIM172 overexpression lines
MIM172 Rev	CATGCACTGGTCTGACTATTCT		
miR172A For	TCTCATTGGCTCTTCTTACTACAA	pK2GW7	Genotyping of 35S:miR172A overexpression lines
miRT35S Rev	ACACATGAGCGAAACCCTATAA		
FUL 1.1 for	CACATAACAAATAATCCATCTTCTCTG	Solyc06g069430	*FUL1* probe for in situ hybridization
FUL 1.1 rev	CGGATATTTTTTCTATACTATGAAT		
FUL 1.2 for	GATGCCACAATATATTGTCTATG		
FUL 1.2 rev	ATGGAATCATACATCAAAATTTATTT		
MBP20.1 for	ATAAAACCTCCCCCCCCAACCC	Solyc02g089210	*MBP20* probe for in situ hybridization
MBP20.1 rev	CCCATCCTCTATCTTTAATGCG		
MBP20.2 for	ATCACATTCTCACCACCAACTTCC		
MBP20.2 rev	CCTAGATCTTTTTAGTACTAAAAAA		
FUL 2.1 for	TGACAACACTTTTGACGAAACCAC	Solyc03g114830	*FUL2* probe for in situ hybridization
FUL 2.1 rev	TTAATTTCTTTCTTTCTTCTTTCTTC		
FUL 2.2 for	ATTAAAACTTAATGAAGTATAAAG		
FUL 2.2 rev	GACAAAACATCCAAAGAGAGGATG		
stem-loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG	*MIR172*	First-strand cDNA synthesis of miR172A
Q-miR172a For	AGCCAGCGAGAATCTTGATGA	*MIR172*	qRT-PCR experiments
Q-universal Rev	GTGCAGGGTCCGAGGT	-	

Q-Pre-miR172_c390 For	AGCTTCACTATCTCTCTATAATCGGT	pK2GW7-miR172A	qRT-PCR experiments
Q-Pre-miR172_c390 Rev	TCATCATATGCAGCATCATCAAGA	pK2GW7-miR172A	
Q-SlACT For	GGTATCCACGAGACTACCTACA	Solyc11g005330	
Q-SlACT Rev	TGCTCATACGGTCAGCAATAC		

Phenotypic analysis.

The number of flowers per plant was measured from the anthesis of the first flower until the last flower opened at the time of the end of flowering. The number of open flowers and new fruits was quantified weekly. The length of the reproductive period was measured in weeks from the initiation of flowering (first anthesis flower) to the last open flower, which determines the onset of proliferative arrest. Total fruit and seed yield per plant was quantified once the plant was arrested and the fruits were ripe. Main sympodia and lateral branches were quantified as actives when they showed one or more flowers at anthesis and therefore are not arrested. For the determinate Micro Tom cultivar (MT-*sp*), the total data produced by both main sympodia, and lateral branches were analyzed. In the case of the indeterminate Micro Tom cultivar (MT-*SP*), given the complexity of its architecture, only flowers and fruits produced on the main axis were measured. The number of independent plants used in each experiment is indicated in every figure.

Statistical analysis

The normal distribution of the data sets was assessed using the Shapiro-Wilk normality test. The normally distributed data was analyzed using the Student's t-test and ANOVA test followed by the HSD Tukey post hoc test. The Mann-Whitney test and Kruskal-Wallis test was used to assess non-normally distributed data. IBM SPSS Statistics 26 (IBM, https://www.ibm.com) served as the tool for the data analysis.

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Conflict of interest

None declared

References

References of chapter 2 are listed at the end of the manuscript.

Supplementary material

The supplementary material of Tables (2.S1-S2) and Figures (2.S1-S7) are placed in the corresponding sections in the thesis manuscript to facilitate the interpretation of the results.

Annex I

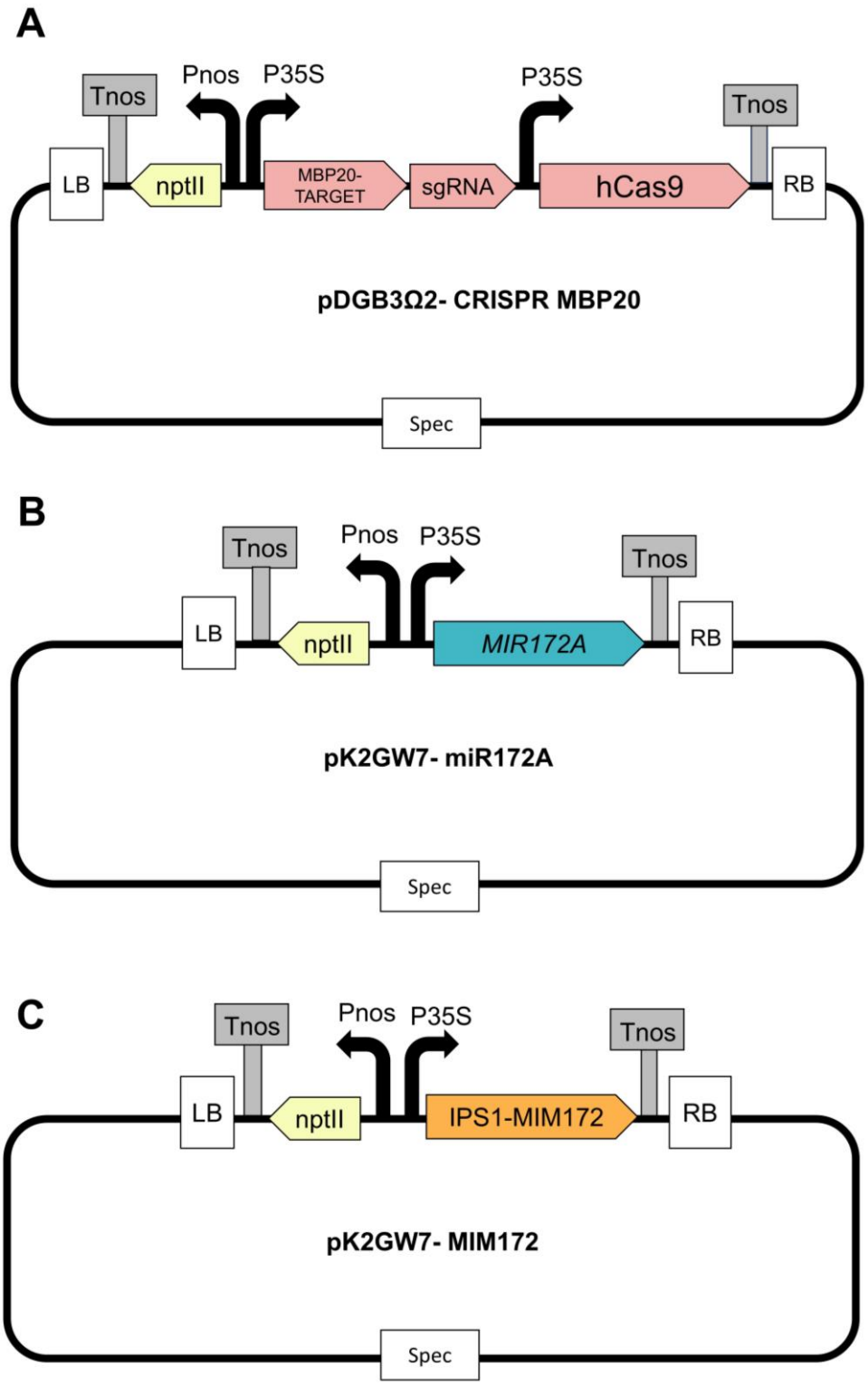

Figure 2.A1. Constructs for the genetic analysis of proliferative arrest in tomato.

A) CRISPR/Cas9 construct for MBP20 gene editing. It includes the human codon-optimized Cas9 (hCas9) under the 35S promoter (P35S) and a guide RNA (sgRNA) specific to MBP20. **B)** Construct for the overexpression of miR172A driven by the 35S promoter (P35S). **C)** miR172 MIMIC construct to modulate SlAP2 gene expression. Panel C shows the 35S:MIM172 module,

where the IPS1 sequence has been adapted to include a reverse-complementary target for miR172A (IPS1-MIM172), acting as a mimic (MIMIC) to inhibit miR172A. A three-nucleotide insertion prevents cleavage and degradation by the miRNA, allowing the MIMIC to bind miR172A, thereby inhibiting its regulatory function and promoting the expression of *SlAP2/SlAP2-like* genes. All modules were cloned into the pK2GW7 vector for expression in tomato, which includes the *nptII* gene for kanamycin resistance driven by the *nos* promoter (Pnos). The 35S promoter (P35S) ensures gene expression, while terminators (Tnos) signal the end of transcription. LB (left border) and RB (right border) refer to the vector's T-DNA borders for plant transformation. Additionally, the plasmid contains the spectinomycin resistance gene (Spec) to facilitate its selection in bacteria.

General Discussion

General Discussion

The end of flowering is a growing field of research in recent years. The understanding of the proliferative arrest (PA) in the model species *Arabidopsis thaliana* has advanced significantly. The most recent studies have focused on the physiological and molecular characterization of this process leading to the identification of genetic factors controlling the end of the life cycle. In addition, how environmental factors interact or control this process in *Arabidopsis* is also being investigated (Balanzà et al., 2018, 2023; González-Suárez et al., 2020; Sadka et al., 2023). On the other hand, comparative studies in other species are still scarce although some studies in *Pisum sativum* have analyzed the role of the seeds in relation to the end of flowering, and the conservation of genetic factors controlling proliferative arrest (Burillo et al., 2024; Martínez-Fernández et al., 2024; Murneek, 1926, 1932).

This thesis provides new data that will help to better understand how different regulatory pathways converge to influence the cessation of meristem activity at the end of flowering. The information obtained in tomato allows to evaluate the conservation of the mechanisms among model organisms, in particular *Arabidopsis thaliana* and other crops such as *Pisum sativum.* Tomato has notable differences from *Arabidopsis* and *Pisum sativum*, such as the production of fleshy fruits rather than dry fruits and a sympodial developmental architecture, in contrast to the monopodial architecture observed in both *Arabidopsis* and *Pisum sativum* (Thouet et al., 2008).

The main conclusion of this thesis is that developing seeds are the major factor triggering proliferative arrest. We performed physiological studies in tomato to determine the role of flowers, fruits and seeds to modulate the end of flowering. Previous studies have suggested that developing fruit/seeds were the main promoters of arrest (Murneek, 1926, 1932), but did not discern the relative importance of fruit or seeds in the process. In this thesis, using parthenocarpic mutants, we precisely identify the seeds as the main factor triggering the proliferative arrest, since seedless mutants maintain their growth through the continuous activation of axillary meristems, giving rise to flowers and consequently fruits. This observation aligns with findings from a parallel study in *Pisum sativum* (pea), where seeds also act as the primary regulators of PA (Burillo et al., 2024), and with Arabidopsis, where seeds also play an important role (Balanzà et al., 2018; Hensel et al., 1994)

In this thesis, in addition to identifying the developing seeds as a source of signals, we also identify auxins (IAA) and abscisic acid (ABA) as key plant hormones in this process.

Our results strongly support the idea that seeds produce a systemic signal, which instructs the meristems to stop producing new flowers and fruits, and therefore the hypothesis of the existence of a *'death hormone'* (Noodén & Leopold, 1988; Proebsting et al., 1977), transmissible by grafting and produced by fruits/seeds, as the signal triggering the arrest. Whether the death hormone is auxin, ABA or other molecule is still unknow, but our work paves the way to further studies to identify its nature.

Despite the common role of seeds found in Arabidopsis and tomato as regulators of PA, the response of the meristem to seeds differs between the two species. In tomato, we have shown that sympodial meristems arrest their proliferative activity once a certain number of fruits and seeds are reached and that it appears that the effect of seeds is proportional and quantitative. Thus, the pruning of 50% of flowers -preventing fruits set- - results in the production of the same additional number of new flowers/fruits before PA. In Arabidopsis, the effect of seeds is not proportional to the timing of PA. Plants that produce up to 30% of the seeds of a fully fertile plant show no effect on PA or total flower production. (Hensel et al., 1994). Only if the production of more than 70% of seeds is prevented, there is a delay in the arrest of inflorescence meristem activity. Thus, this suggests that in both species, there is a seed effect that triggers proliferative arrest, and in tomato it is the sympodial meristems and not the inflorescence meristem that integrates these signals.

Initially, in Arabidopsis, the control of proliferative arrest was described as a systemic process in which the presence of fruits triggers a synchronized cessation of proliferative activity in all inflorescence meristems (Hensel et al., 1994) . It was considered a global process, as the arrest signals, driven by fruit production, affect not only the meristems closest to the fruits but also those furthest away. The result is the simultaneous interruption of flowering throughout the plant, indicating a broad and systemic regulation of meristem activity. Following this study, the concept of global proliferative arrest (GPA) was defined to refer to this process related to the end of flowering in monocarpic plants (Hensel et al., 1994)

In contrast, recent studies have reviewed the global or local nature of proliferative arrest. The works by Ware et al. (2020) and Walker et al. (2023) argue in favour of the localized nature of proliferative arrest in *Arabidopsis*. Their findings suggest that fruit position plays a critical role in this process, with fruits closer to the shoot apical meristem (SAM) exerting a greater influence on the cessation of meristem activity, and that the arrest of the meristems in different inflorescence branches can be uncoupled. This means that the proliferative arrest is not globally coordinated, as initially suggested by Hensel, but

rather localized, depending on the proximity of the fruits to the meristem(Walker et al., 2023).

Regarding tomato, our observations suggest that the proliferative arrest seems to be global, as fruit removal triggers a global activation of axillary meristems, and once the total number of fruits reaches a critical number, the end of flowering takes place. Our results show that seed production globally influences the activation of sympodial and axillary meristems, causing a synchronized cessation of meristem activity in all sympodial units.

The apparent divergence of the coordination of meristem arrest can be discussed in the context of the different inflorescence architecture in Arabidopsis and tomato. As previously mentioned, It has been described that proliferative arrest in *Arabidopsis* occurs in two phases: first, inflorescence meristem arrest, followed by the developmental block of floral bud development, or floral arrest, and that only this latter appears to respond to auxin exported from proximal fruits (Walker et al., 2023; Ware et al., 2020). This two-step mechanism highlights a coordinated regulation of arrest phases involving both local auxin signaling and global developmental signals. Thus, in Arabidopsis, floral arrest is linked to the cessation of activity in the indeterminate inflorescence meristem (IM), which has no equivalent in tomato.

In tomato, all inflorescence meristems are determinate and there is no obvious separation between sympodial and floral arrest phases. This suggests that proliferative arrest in tomato behaves more like a one-step sympodial arrest, rather than following the two-step arrest mechanism described in Arabidopsis. In tomato it appears that it is the fruits at early developmental stages that produce phytohormones such as IAA or ABA. Our results suggest that meristems detect these phytohormones globally and do not depend on relative position with respect to the fruit. For this reason, it is possible to speculate that the relative importance of factors triggering arrest in both species, and even the nature of the signalling molecules involved, are nor fully equivalent.

This comparison highlights the need to further explore how different species regulate meristem activity at the end of flowering, responding to external and endogenous cues.

In chapter 2 we addressed the conservation of the genetic networks that control proliferative arrest in tomato. We chose to focus on the main modulators of proliferative arrest described so far, the miR172-SlAP2-SlFULs module (Balanzà et al., 2018), characterizing the phenotypic effect of modifications in the activity of these factors.

miR172 conserves its role in the regulation of proliferative arrest in tomato by repressing the activity of *SlAP2/AP2-like* genes, which promotes meristem activity. In Arabidopsis, miR172 down-regulates *AP2*, triggering meristem arrest. This functional conservation suggests that miR172-mediated down-regulation of AP2 contributes to the regulation of meristem activity is a conserved regulatory mechanism in these species.

However, our data suggest that constitutive expression of miR172 alone is not enough to induce proliferative arrest (PA) in the absence of seed development, as shown by the pruned *35S::miR172A* lines where flower production continued in the absence of seed development. These lines show a decrease in the flower production rate compared to the pruned wild type, but without undergoing proliferative arrest. This suggests that, although miR172A represses *SlAP2* activity and overexpression of miR172A increases this repression, it is not sufficient for the plants to reach the end of flowering in the absence of seeds. This points to a more relevant role of seeds in triggering proliferative arrest than miR172. Seed-derived signals would be acting as the main signal, and miR172 would act as a modulator of this arrest by an independent pathway, as it contributes to the regulation of meristem activity but does not compensate for the total absence of seeds (Balanzà et al., 2018; Martínez-Fernández et al., 2020, 2024). An alternative explanation could be that in miR172-overexpressing plants, meristems are more sensitive to seed-derived signals. Maybe #OE-miR172 plants have meristems that are more sensitive to the perception of fruit-derived signals and these cues, despite being weaker due to the reduction in the number of seeds of these fruits, could be regulating *SlAP2/SlAP2-like* activity.

The second component of the miR172-SlAP2-SlFUL module involves *FUL* homologues. In *Arabidopsis*, the FUL-AP2 pathway is an age-dependent mechanism controlling the floral transition and meristem arrest, with *FUL* acting as a single functional gene (Balanzà et al., 2018). In contrast, in *Pisum sativum*, there are two *FUL* homologues (*PsFULa* and *PsFULb*) that appear to function redundantly in meristem regulation (Martínez-Fernández et al., 2020, 2024).

However, the scenario in tomato is complex, as there are four *FUL* homologues (*SlFULs*): *FUL1*, *FUL2*, *MBP10* and *MBP20*. In *Chapter 2*, we show that the function of *MBP20* in tomato appears to diverge from that of its homologues in Arabidopsis and *Pisum sativum*. We show that *mbp20* mutants exhibit early proliferative arrest (PA), a phenotype opposite to that observed in Arabidopsis and *Pisum sativum ful* mutants, suggesting that *MBP20* has undergone neofunctionalisation, acquiring a function distinct from that of the other *SlFUL* homologues. However, this idea has to be taken with

caution, since MBP20 belongs to the AGL79 clade, which has not been characterized for its role in proliferative arrest in detail yet.

. In tomato, our results suggest that the miR172-AP2 module plays a conserved and more determinant role in the regulation of proliferative arrest compared to *MBP20*. Further analyses of all *SlFULs* homologs are needed to understand their contributions and how they fit into the genetic network controlling this process. While we have primarily focused on *MBP20,* a FUL homolog from the *euFULII clade*, which promotes meristematic activity and thus prolongs the reproductive phase, the roles of other *FUL* genes remain unexplored. However, the early arrest observed in *mbp20* mutants indicates that *MBP20* is functionally important. Future research should explore whether other *FUL* homologues contribute to meristem regulation in tomato to clarify their interactions with the *miR172-AP2* pathway.

The findings of this thesis (Figure G.D.1) have important implications for crop improvement. Understanding the genetic and hormonal mechanisms that regulate PA can help to develop tomato varieties with longer fruiting periods, leading to increased yields. Manipulation of genetic regulators such as miR172 or *SlFULs* as well as seed-derived signals such as auxins or ABA, could make possible to modulate the length of the reproductive phase in tomato.

Thus, the knowledge gained in this thesis paves the way to design biotechnological strategies to modulate the duration of the reproductive phase in tomato and other crops of interest.

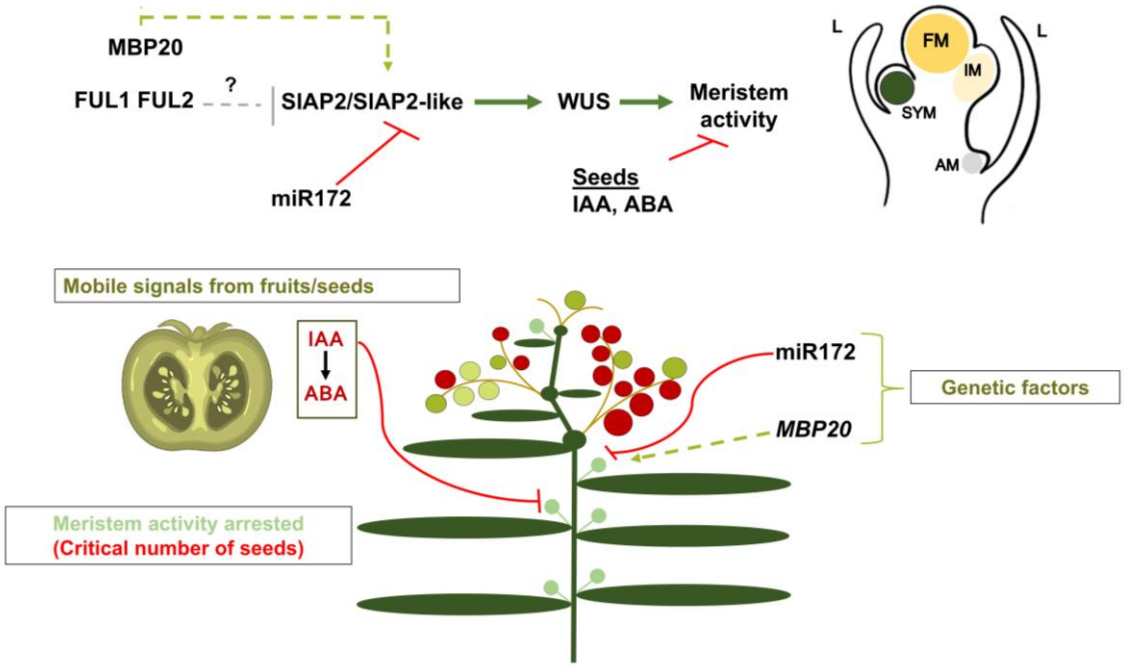

Figure G.D.1. **Working model illustrating the regulation of proliferative arrest (PA) in tomato (*Solanum lycopersicum*).** Green arrows indicate factors that promote meristem activity, while red arrows represent inhibitory signals. The thickness of the arrows corresponds to the strength and certainty of each regulatory relationship based on current evidence. Seed-derived signals, particularly auxins (IAA) and abscisic acid (ABA), play a central role in repressing meristem activity. The miR172-AP2 module, conserved between tomato and *Arabidopsis*, also regulates PA by downregulating *AP2-like* genes to promote early arrest.

Conclusions

Conclusions

Chapter 1

1. Seed production is the main factor triggering proliferative arrest in tomato plants, as evidenced by the continuous growth observed in seedless mutants. This highlights the quantitative role that seeds play in controlling the lifecycle in *Solanum lycopersicum*.
2. Proliferative arrest in tomato occurs in a global and synchronized process in all sympodial units once a critical number of seeds is reached.
3. Auxins (IAA) are a hormonal signal produced by fruits/ seeds that regulates the cessation of meristem activity. IAA exerts a decisive influence on meristem proliferation, Although ABA also plays a role, IAA is more directly linked to the suppression of meristem function, making it the critical hormone driving the cessation of meristem activity.

Chapter 2

1. The miR172-AP2 genetic module controls proliferative arrest in tomato, similarly to what has been described in Arabidopsis, where AP2 is required for meristem proliferation and miR172 represses AP2 activity promoting PA.
2. *MBP20*, a tomato homolog of the *FUL* gene, is essential for maintaining meristem activity. Mutations in *MBP20* lead to early termination of flowering, underlining its role in maintaining meristem activation throughout the reproductive phase of the plant.
3. In both determinate and indeterminate tomato cultivars, *MBP20* and *miR172* regulate sympodial meristem longevity and axillary branching. Genetic manipulation of key factors such as miR172 and *MBP20* could enhance fruit yield by extending the reproductive phase.

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Acknowledgements

[2]

[2] Metamorphosis of the Swallowtail butterfly (*Papilio machaon*). Image attributed to Jens Stolt.

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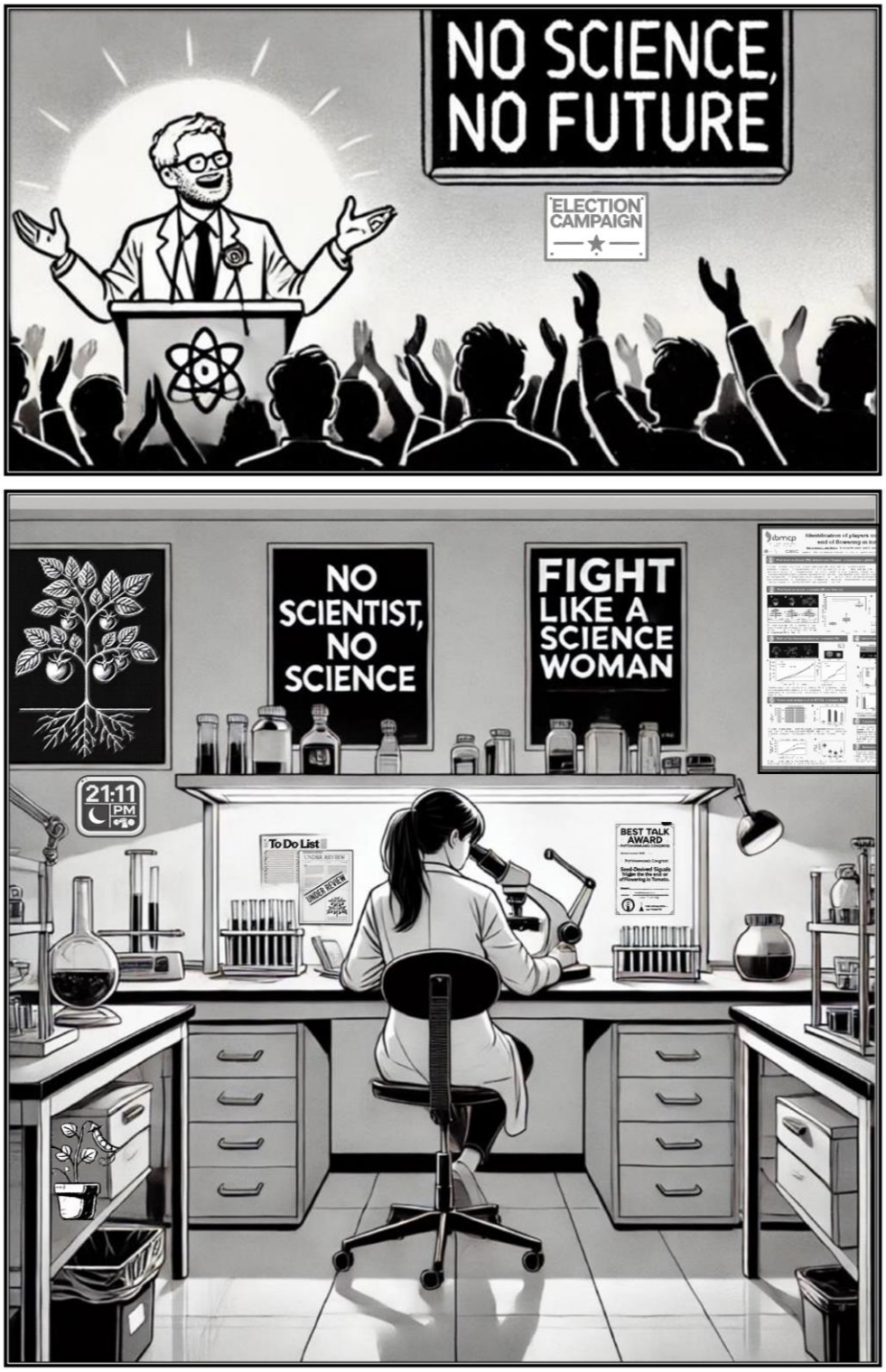

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