The role of *FRUITFULL*-like genes in the tomato flowering regulatory network

Xiaobing Jiang

Propositions

1. *FRUITFULL*-like genes regulate reproductive development in tomato.
   (this thesis)

2. Cytokinin plays a crucial role in flowering regulation.
   (this thesis)

3. Even the protocols for well-established techniques will not work in every lab.

4. Current attention on orphan crops is too limited.

5. Social media are increasing the intergroup prejudice.

6. Someone who is good at cooking will never gain weight.

7. An apparatus that can monitor the work-life balance would be highly appreciated by many.

Propositions belonging to the thesis:
The role of *FRUITFULL*-like genes in the tomato flowering regulatory network

Xiaobing Jiang
Wageningen, 2 March 2022

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CHAPTER 1

General Introduction

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Tomato is the second most important vegetable crop worldwide. Because of its great popularity, plant scientists and breeders have made enormous efforts to improve fruit quality and yield to meet market needs. Plant architecture is an important morphological feature that determines reproductive performance and has therefore been a crucial aspect during crop domestication. Tomato plant architecture is mainly defined by the degree of inflorescence branching, shoot compactness, and determinacy, which together determine the yield of mechanical harvesting. Studying the molecular basis of these developmental processes will increase the understanding of the different traits and provide new resources for crop improvement.

The plant shoot apical meristem (SAM) is the basis of the formation of all above-ground parts, including leaves, stems, inflorescences, and flowers. These organs result from the dynamic growth of different meristems, and are either initiated directly from the SAM, such as leaves or, after reproductive transition, from derived meristems such as the floral meristem (flower) or grow from newly formed meristems, such as the axillary meristem (branches). Many genes underlying reproductive development are active in more than one type of meristem, and thus influence more than one trait, such as flowering time and plant architecture. Below, an overview is presented on the molecular regulation of plant reproductive development, related to agricultural relevance and mainly focusing on tomato.

SAM growth dynamics

All aerial parts of the plant are generated from the SAM, which is initiated during embryogenesis and consists of undifferentiated cells. These stem cells have been subdivided into distinct zones based on cytoplasmic densities and cell division rates (Steeves and Sussex, 1989). The central zone (CZ) in the centre of the SAM acts as a reservoir of stem cells that replenish both the peripheral zone (PZ) and rib zone (RZ). The PZ produces lateral organs, while the RZ generates the outermost layers of the stem (Meyerowitz, 1997). Below the CZ, a group of cells are required to organize and maintain the stem cell population and are therefore referred to as the organizing centre (OC) (Mayer et al., 1998; Fuchs and Lohmann, 2020). Size and structure of the SAM are regulated by an interplay between cell division and cell differentiation that is controlled by integrated ligand-receptor networks, hormone pathways, and transcriptional regulators. Crucial in the regulatory network is a feedback loop between the peptide *CLAVATA3* (*CLV3*) and the transcription factor *WUSCHEL* (*WUS*) that coordinates stem cell proliferation with differentiation. The small, secreted peptide *CLV3*, which is expressed in the CZ and acts as a ligand to the receptors, including the receptor-like kinase *CLV1* and receptor-like protein

CLV2, represses the homeodomain transcription factor *WUS,* thereby restricting its expression to the underlying OZ, and promoting cell differentiation for organ initiation (Fletcher et al., 1999; Brand et al., 2000; Schoof et al., 2000; Rojo et al., 2002; Fiers et al., 2005; Müller et al., 2008; Hu et al., 2018). At the same time, WUS moves from OZ to CZ via plasmodesmata to activate *CLV3* expression, promoting cell proliferation for stem cell identity (Yadav et al., 2010; Yadav et al., 2011; Perales et al., 2016). The *clv* mutants show an enlarged SAM due to the accumulation of stem cells that in turn delay organ initiation (Clark et al., 1995; Clark et al., 1997; Jeong et al., 1999). Loss of *WUS* function impairs the specification of the central stem cells, prematurely arresting organ formation (Laux et al., 1996). Thus, *CLV3* regulates cell division to restrict SAM size by transcriptional restriction of *WUS* expression (Aichinger et al., 2012; Somssich et al., 2016).

Knotted1-like homeobox (KNOX) transcription factors are the central players in SAM maintenance by repressing cell differentiation. The Arabidopsis genome carries four Class I KNOX genes: *SHOOT MERISTEMLESS* (*STM*), *BREVIPEDICELLUS* (*BP*), *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2* (*KNAT2*) and *KNAT6* (Barton and Poethig, 1993; Lincoln et al., 1994; Pautot et al., 2001). These genes function distinctly but partially redundantly in stem cell maintenance and organogenesis. Among them, *STM* is the first KNOX gene expressed during early embryogenesis and accumulates throughout the SAM. Loss of *STM* results in failure to specify and maintain the SAM (Clark et al., 1996; Endrizzi et al., 1996; Long et al., 1996). *STM* plays a role by binding to the promoter of *CLV3* to activate its expression, or through physical interaction with *WUS* to enhance *WUS* binding to *CLV3* promoter for its expression (Brand et al., 2002; Su et al., 2020). Generally, KNOX proteins promote meristem activity by simultaneously repressing gibberellin (GA) biosynthesis and activating cytokinin (CK) at the shoot apex (Jasinski et al., 2005; Yanai et al., 2005).

Crosstalk of the plant hormones cytokinin (CK) and auxin is another important aspect of the regulatory network that controls shoot meristem development. Defects in CK synthesis or perception reduce SAM size, indicating a positive role for CK in meristem development (Werner et al., 2003a; Higuchi et al., 2004; Werner and Schmülling, 2009). CK signalling is transmitted via the type-B ARRs, which directly bind to the WUS promoters to promote its expression (Meng et al., 2017; Wang et al., 2017; Zubo et al., 2017; Xie et al., 2018), and via the type-A ARRs, which are negative regulators of CK signalling in the RZ and repressed by WUS (Leibfried et al., 2005; Gordon et al., 2007; Zhao et al., 2010). However, auxin plays an antagonistic role with CK on SAM activities, involving the coordination of signalling, biosynthesis, and transport pathways (Chickarmane et al., 2012; Truskina and

Vernoux, 2018; Kurepa et al., 2019). Both hormones directly converge on *ARR7*/*15* that are induced by CK while being repressed by auxin (Zhao et al., 2010).

The balance of cell division and cell differentiation in the meristem, controlled by the genetic interactions described above, can be quantitatively tuned for crop improvement. Dependent on the crop, boosting yield can be achieved by remodelling of plant architecture, increasing the number of seeds and/or the size of the fruits. Genes controlling meristem size can be quantitively modified for agricultural practices. For example, the *CLV*-*WUS* pathway was first defined in Arabidopsis, but has been found conserved in flowering plants such as maize, rice and tomato (Fletcher, 2018). In tomato, disruption in *CLV*-*WUS* circuit genes increases the fruit size. The partial loss of *SlCLV3* (*fasciated, fas*) causes increased locule number that results in bigger fruits and higher yields, and the *slclv3* null mutant develops severely enlarged meristems and many more fruit locules (Xu et al., 2015). Similarly, the *locule number* (*lc*) mutation caused by two SNPs in a putative cis-regulatory element located 1080 bp from the 3' end of tomato *WUS* (*SlWUS*), presumably enhances *SlWUS* expression and shows a mild increase in locule number (Muños et al., 2011; van der Knaap et al., 2014; Xu et al., 2015).

Flowering time regulation

Appropriate timing of flowering is critical for plant reproductive development. During post-embryonic development of flowering plants, the SAM first produces vegetative organs, such as stem and leaves. A combination of internal and external signals can induce the switch to reproductive growth, which is accompanied by changes in the SAM. The SAM size remains roughly the same during the vegetative stage but shifts from a flat top to a domed shape upon the transition to flowering. The SAM doming is usually associated with floral transition in both monocot and dicot species and. Although it is unclear why most plant meristems dome during floral transition, a prime and universal booster is the so-called 'florigen' (Shalit et al., 2009; Pin and Nilsson, 2012).

The transition to flowering is regulated by over 300 genes at genetic and epigenetic levels in *Arabidopsis*, such as transcriptional regulation, alternative splicing, non-coding RNA-based regulation, and chromatin modifications (Dennis and Peacock, 2007; Khan et al., 2014; Bouché et al., 2016; Campos-Rivero et al., 2017). Many of these genes are transcription factors that act as floral activators or repressors in multiple regulatory pathways, including the vernalization-, autonomous-, GA-, age-, ambient temperature- and photoperiod-pathways (Fornara et al., 2010; Andrés and Coupland, 2012). These pathways regulate plant flowering individually in response

to environmental or internal cues, and their output generally converges on two master integrators of flowering signals, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*). *FT*, encoding the florigen, is expressed and translated in the leaves as a photoperiodic signal mediated by signaling input from CONSTANS (CO), and the FT protein then moves to the SAM through the phloem to promote floral development (Turck et al., 2008). It is regulated by a number of upstream factors in the leaves, including (ambient) temperature and day-length signals (Fornara et al., 2010). The MADS-box transcription factor *SOC1* is activated by the GA- and age pathway but repressed by the ambient temperature and vernalization pathways (Fornara et al., 2010; Lee and Lee, 2010). *SOC1* directly responds to leaf-produced FT at the apex and its activation during long days requires FT. The activation of flowering integrators, in turn, activates the floral meristem identity genes, *LEAFY* (*LFY*) and *APETALA1* (*AP1*), changing vegetative meristem (VM) to inflorescence and floral meristem. The floral transition is prevented by the floral repressors on floral integrators, such as *FLOWERING LOCUS C* (*FLC*), *TERMINAL FLOWER 1* (*TFL1*), and *SHORT VEGETATIVE PHASE* (*SVP*) (Fornara et al., 2010). Notably, a repressor complex that consists of *FLC* and *SVP* suppresses *SOC1* expression during the vegetative stage, whereas the autonomous-, GA-, ambient temperature- and vernalization pathways down-regulate *FLC* and *SVP*, and thereby derepress *SOC1* during the floral transition (Samach et al., 2000; Moon et al., 2003; Lee et al., 2007). Despite distinct functions, the roles of the Arabidopsis key flowering regulators appear to be conserved in many other species even when these have different flowering strategies. Also, the function of key genes in the separate pathways is often conserved. For example, homologs of *CO* act as photoperiod-dependent regulators of *FT* homologs in rice (Hayama et al., 2003), sorghum (Yang et al., 2014), barley (Mulki and von Korff, 2016), and potato (Abelenda et al., 2016).

Many genes play a role in different flowering pathways, thereby influencing more than one developmental process, such as the reproductive phase change and floral meristem identity. Therefore, loss of these genes will affect not only flowering, but also floral organ development, inflorescence development and/or plant architecture. An example of such a multifunctional regulator is the MADS-domain transcription factor *FRUITFULL* (*FUL*), which is involved in the photoperiod-, ambient temperature- and age pathways. In response to long days, *FUL* promotes flowering in the shoot apex in a partially redundant manner with *SOC1*, where both are activated by FT (Teper-Bamnolker and Samach, 2005; Yoo et al., 2005; Melzer et al., 2008b). *FUL* is one of the most responsive genes to the SQUAMOSA PROMOTER BINDING LIKE (SPL) proteins in the age pathway, and to

miR156/SPL3 and FT in the ambient temperature pathway (Wang et al., 2009; Yamaguchi et al., 2009; Kim et al., 2012). In addition to promoting flowering, *FUL* plays roles in FM identity specification together with *AP1*, in carpel initiation and development, secondary growth, and the termination of the inflorescence meristem (Hempel et al., 1997; Gu et al., 1998; Ferrándiz et al., 2000; Melzer et al., 2008b; Balanzà et al., 2018). *FUL* belongs to the *APETALA1*/*FRUITFULL* (*AP1/FUL*) subfamily of the MADS-box transcription factor gene family. In the core eudicots, this subfamily consists of three clades: *euAP1*, *euFULI* and *euFULII* (Litt and Irish, 2003). Some species have one representative in each clade, but due to lineage-specific duplications, many species also harbour more genes per clade. For example, the tomato genome only contains one *euAP1* gene, but two paralogs in both the *euFULI* and *euFULII* clades (Figure 1). In species with multiple paralogs per clade, sub-functionalization may have occurred during evolution, resulting in paralogs with only a subset of the original functions. In a wide range of angiosperm species, *FUL*-like genes regulate flowering (Ferrándiz et al., 2000; Berbel et al., 2012; Pabón-Mora et al., 2012; Ping et al., 2014; Jaudal et al., 2015; Jia et al., 2015; Li et al., 2019a; Zhang et al., 2021) and fruit development (Gu et al., 1998; Jaakola et al., 2010; Bemer et al., 2012; Pabón-Mora et al., 2013; Zhao et al., 2019). In tomato (*Solanum lycopersicum*), the *euFULI*-clade genes *S. lycopersicum FUL1* (*SlFUL1*) and *SlFUL2* play important roles in fruit development and ripening (Bemer et al., 2012; Shima et al., 2013; Wang et al., 2019b), but whether they are also involved in flowering in tomato is unclear.

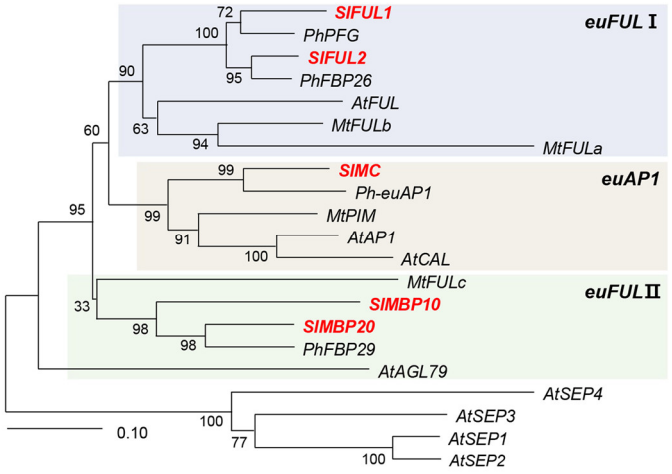


Figure 1. Phylogenetic analysis of *AP1/FUL*-like genes of *A. thaliana* (*At*), *S. lycopersicum* (*Sl*), *P. hybrida* (*Ph*), and *M. truncatula* (*Mt*). The phylogenetic tree was constructed using the neighbor-joining method with protein sequences. Bootstrap values

estimated with 1000 reiterations are indicated above the branches. Accession numbers: *AtFUL*, AT5G60910; *AtAP1*, AT1G69120; *AtAGL79*, AT3G30260; *AtCAL*, AT1G26310. *AtSEP1*, AT5G15800; *AtSEP2*, AT3G02310; *AtSEP3*, AT1G24260; *AtSEP4*, AT2G03710; *SlFUL1*, Solyc06g069430; *SlFUL2*, Solyc03g114830; *SlMBP10*, Solyc02g065730; *SlMBP20*, Solyc02g089210; *SlMC*, Solyc05g056620; *PhFBP29*, AF335245.1; *PhFBP26*, AF176783.1; *Ph-euAP1*, MK598839; *PhPFG*, AF176782.1; *MtFULa*, Medtr2g461760; *MtFULb*, Medtr4g109830; *MtFULc*, Medtr7g016630; *MtPIM*, Medtr8g066260. The tomato proteins are indicated in red.

Tomato flowering regulation

Tomato plants comprise a primary shoot and sympodial shoots in which the flowering regulation differs. After initiation of 6~12 leaves (dependent on the cultivar), the shoot apex transitions to flowering, which is associated with apical doming of the VM (Figure 2). The VM then develops into a transition meristem (TM) and terminates into a floral meristem (FM) that forms a new meristem on its flank to initiate inflorescence development. The primary vegetative apex is terminated by the inflorescence (primary shoot), whereas the vegetative growth is resumed from the sympodial meristem (SYM) that arises from the axil of the youngest leaf primordium. After the formation of a distinct number of leaves (usually three), the sympodial apex makes the transition to reproductive development, forming an inflorescence similar to the primary shoot. The tomato plant thereby generates a sympodial shoot, where the vegetative and reproductive phases alternate regularly. The flowering time of the primary shoot is usually indicated by the number of leaves to the first inflorescence as this is rather stable in changing environments, and the sympodial flowering time is measured by the number of leaves between inflorescences. Although the processes of primary shoot flowering and sympodial shoot flowering are very similar, small differences are present in the regulatory network.

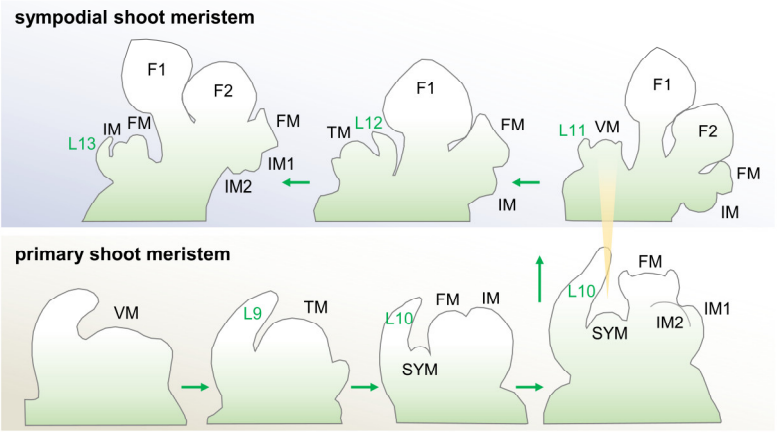


Figure 2. Representative meristem growth dynamics during floral transition and inflorescence development. VM: vegetative meristem, TM: transition meristem, FM: floral meristem, IM: inflorescence meristem, F: flower, SYM: sympodial meristem, L: leaf.

Defects in flowering result from impaired specification of the IM or FM. So far, the tomato flowering pathway is not fully resolved, but many regulatory components have been uncovered by mutant studies. These regulators are mostly the orthologs of Arabidopsis flowering genes and determine flowering in the primary shoot and sympodial shoot. They include *SINGLE FLOWER TRUSS* (*SFT*, ortholog of *FT*), a leaf-produced mobile florigen that induces flowering in both primary and sympodial shoots (Molinero-Rosales et al., 2004; Lifschitz and Eshed, 2006; Lifschitz et al., 2006), *SELF-PRUNING* (*SP*, ortholog of *TFL1*), *JOINTLESS* (*J*, homolog of *SVP* and *AGL24*), *ANANTHA* (*AN,* homolog of *UFO*), and *FALSIFLORA* (*FA*, homolog of *LFY*). Together with other genes, such as *UNIFLORA* (*UF*) and *BLIND* (*BL*), they control flowering in an epistatic or synergistic way. For instance, the single *fa* and *sft* mutants display a delay in flowering time, but the transition to flowering is completely blocked in the double knock-out of both genes (Molinero-Rosales et al., 2004; Quinet et al., 2006a). These genes control meristem fate and their functional disruptions lead to failed IM or FM establishment, and thus, defected flowering. By contrast, loss of *TERMINATING FLOWER* (*TMF*), encoding an ALOG (Arabidopsis LSH1 and Oryza G1) family gene, causes much earlier flowering due to de-repression of the *AN* gene and precocious adoption of floral identity (MacAlister et al., 2012b). Gene network analysis revealed that the floral transition of primary shoot and sympodial shoots are regulated slightly differently, probably due to differences in the expression of a few genes (Quinet et al., 2006a; Thouet et al., 2012a). The *SFT*-antagonist *SP* is the most prominent one, controlling the sympodial flowering time. The repressor SP is strongly expressed in the

vegetative sympodial meristem, but is not expressed in the primary shoot VM (Pnueli et al., 1998; Thouet et al., 2008).

The environmental stimuli that affect tomato flowering are mainly temperature and light. Classical studies have shown that low temperature induces early flowering during a nine-day sensitive phase after cotyledon expansion (Lewis, 1953; Calvert, 1957). The changes of the quantity, quality and duration of light interactively regulate flowering in tomato. Although tomato is generally considered as a photoperiod insensitive species, wild species are short-day plants and some cultivars flower late in long days (Wittwer, 1963; Binchy and Morgan, 1970; Lifschitz and Eshed, 2006). Upon domestication, the day-length responses of tomato cultivars have reduced. It is the *SFT*-homolog, *SELF-PRUNING 5G* (*SP5G*), that mediates the loss of day-length-sensitive flowering by reducing long-day response and another *SFT* paralog, *FT*-Like 1 (*FTL1*), which determines tomato response to short-day (Carmel-Goren et al., 2003; Soyk et al., 2017b; Song et al., 2020). Loss of *SP5G* causes rapid flowering while *FTL1* loss delays flowering in tomato (Soyk et al., 2017b; Song et al., 2020). Furthermore, a lower R:FR ratio delays tomato flowering via increased *SP5G* expression (Cao et al., 2018; Kim et al., 2019).

Inflorescence architecture development

The inflorescence architecture is a major determinant of plant fitness in nature. Flowering plants have evolved diverse inflorescence morphologies that can be mainly classified as determinate (cyme type) or indeterminate (raceme and panicle types), depending on whether the primary IM terminates into a flower or not. Upon floral transition in indeterminate inflorescences, the SAM grows indefinitely to generate either flowers and form a raceme-type inflorescence such as in *Arabidopsis* (Figure 3A) or to generate branch meristems, which produce branches with flowers, resulting in a panicle-type inflorescence such as in rice. In contrast, the SAM in determinate species becomes an IM that acquires FM identity to develop a terminal flower. The determinate class includes species with the simplest architecture, such as Tulip, and more complex forms, for instance, the cyme type in some Solanaceae species, where the FM gives rise to a lateral IM before flower termination, so that the successive IM-FM maturations produce a multiflowered inflorescence (Figure 3B). Therefore, it is the SAM determinacy that determines the inflorescence architecture (Weberling, 1992; Prusinkiewicz et al., 2007).

The activity and developmental trajectories adopted by axillary meristems (AMs) add another layer of inflorescence complexity. The AMs formed in the leaf axils of inflorescences can remain vegetative, giving rise to branches, or adopt IM/FM fate.

The branches display internode elongation, produce flowers or secondary branches, and can be indeterminate (*Arabidopsis*) or determinate (e.g., pea). Thus, flowers can be directly formed either from the primary inflorescence axis (simple types), or in secondary or higher order axes (compound types), depending on the AM's fate. Both primary IM and AM determinacy are repressed by *TFL1*, but promoted by floral inducers such as *LFY* and *AP1* (Zhu and Wagner, 2020). *tfl1* mutants often generate a determinate inflorescence with a terminal flower, similar to plants overexpressing *FT* (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1997; Ohshima et al., 1997; Kardailsky et al., 1999; Kobayashi et al., 1999; Lee et al., 2019). Although *TFL1* and *FT* play antagonistic roles in IM determinacy, *FT* function appears more important in this process as in *Arabidopsis*, the normal inflorescence is converted to a terminal flower regardless of *TFL1* level when the *FT* levels are very high (Lee et al., 2019).

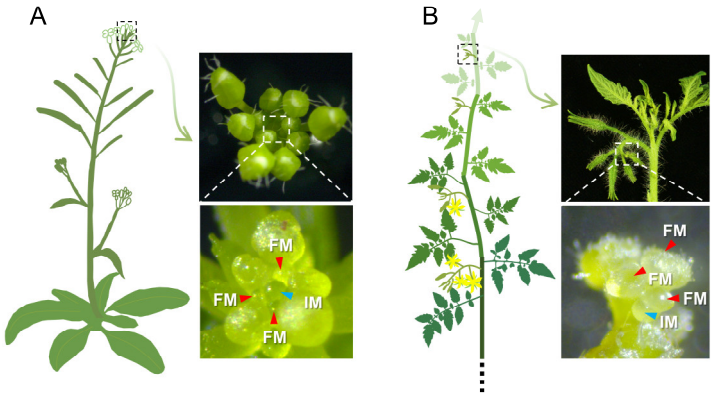


Figure 3. The difference between the monopodial indeterminate Arabidopsis inflorescence (A) and the sympodial determinate tomato inflorescence. (B) IM: inflorescence meristem (green arrows). FM: floral meristem (red arrows).

Diverse other regulators, such as key flowering genes and hormones, have an influence on inflorescence architecture through the regulation of meristem identity (Han et al., 2014a; Teo et al., 2014). The flowering genes mediate the switch from vegetative growth to reproductive development by repressing genes that maintain the vegetative phase, or by activating floral meristem identity genes that regulate on their turn the floral organ identity genes. The classic paradigm controlling the inflorescence branching pattern is the antagonistic interaction between the shoot meristem identity gene *TFL1* and the floral meristem identity genes *LFY* and *AP1*. Loss of *TFL1* function converts the IM into an FM, resulting in a determinate inflorescence (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bowman et

al., 1993). By contrast, mutations in *LFY* or *AP1* allow ectopic and/or upregulated *TFL1* expression, which reverts the FM into IM. A knock-out of both *AP1* and its paralog *CAULIFLOWER* (*CAL*) in Arabidopsis enhance the mutant phenotype, resulting in a cluster of proliferating inflorescence meristems, and the loss of floral meristem fate is even more severe when also their close homolog *FUL* is mutated in the *ap1cal* mutant background, suggesting an additional role for *FUL* in the specification of the FM (Bowman et al., 1993; Liljegren et al., 1999; Ratcliffe et al., 1999; Ferrándiz et al., 2000). The mode-of-action of *AP1* has been unravelled with detailed target gene analyses, which revealed that it acts as a repressor of IM identity genes such as TFL1 in the early phase, and subsequently activates floral organ identity genes (Kaufmann et al., 2010). Other flowering genes, such as *SOC1*, *AGL24* and *SEP4* contribute to *TFL1* suppression to confer FM identity (Kaufmann et al., 2010; Liu et al., 2013). Additional key regulators have been identified in other species, such as rice, which serves as the monocot model to investigate the control of inflorescence architecture. CKs have a common positive effect on inflorescence branching through promoting IM activity. Besides, CKs promote *WUS* expression through both CLAVATA-dependent and -independent pathways and suppress *CLV1* and *CLV3* expression (Leibfried et al., 2005; Gordon et al., 2009). Elevated CK homeostasis and signalling increase the inflorescence complexity and vice versa. For example, reduced expression of rice *CYTOKININ OXIDASE/DEHYDROGENASE* 2 (*OsCKX2*) causes CK accumulation in IMs, promoting panicle branching and higher grain productivity, whereas loss of the CK-activating gene *LONELY GUY* (*LOG*) results in compromised panicle development (Ashikari et al., 2005; Kurakawa et al., 2007).

In agriculture, these genetic targets have been used to favour inflorescence modifications, especially inflorescence branching, to increase flower and grain production during crop domestication and improvement. A striking example are the flower heads of broccoli and cauliflower that carry a nonsense mutation in the MADS-box gene *BoCAL, which* arose during domestication (Kempin et al., 1995; Purugganan et al., 2000). Additionally, increased inflorescence branching substantially contributes to an increase in yield for cereal crops, including barley (Ramsay et al., 2011), maize (Eveland et al., 2014), rice (Huang et al., 2009), and wheat (Boden et al., 2015).

Tomato inflorescence development

Species of the Solanaceae family show a great inflorescence diversity ranging from solitary flowers to highly branched structures. The domesticated tomato and wild

ancestor *Solanum pimpinellifolium* produce a single-branched inflorescence with multiple flowers. Although several wild tomato relatives develop weakly branched inflorescences, they have been underexplored, largely because of genetic incompatibilities (Lemmon et al., 2016). The zigzagged inflorescence pattern in most tomato varieties, known as “tomatoes-on-the-vine”, results from a sequential IM-to-FM maturation during inflorescence development. This progressive IM-to-FM maturation defines a time window during which a new lateral IM can be formed, and delays in acquiring FM fate allow additional IMs to be formed, leading to highly branched inflorescences (Lippman et al., 2008a; Park et al., 2012). Thus, FM determinacy fundamentally determines tomato inflorescence architecture, and several genes have been identified that delay the establishment of the determinate FM (also defined as ‘delay in FM maturation’(Park et al., 2012)).

Transcription factors with known roles in flowering define meristem maturation schedules, which underlie architectural variation in the Solanaceae (Lemmon et al., 2016). Although mutations in flowering genes often affect more than one type of meristem, distinct effects on a particular meristem type or function are commonly observed. Prominent examples include the late flowering *sft* mutant that produces either a solitary flower or inflorescences that revert to vegetative growth after producing a few flowers (Molinero-Rosales et al., 2004; Lifschitz and Eshed, 2006; Lifschitz et al., 2006; Quinet et al., 2006b). Loss of other genes, such as the MADS-box genes *MACROCALYX* (*MC*, ortholog of *AP1*) and *J,* lead to mildly delayed flowering and inflorescences that partially or completely revert to vegetative growth (Vrebalov et al., 2002; Szymkowiak and Irish, 2006; Yuste-Lisbona et al., 2016b). These phenotypes arise probably because the mutant meristems fail to adopt or maintain FM identity. At the other hand, combined null mutations of two SEPALLATA-like MADS-box genes, *JOINTLESS 2* (*J2*/*SlMBP21*) and *ENHANCER OF JOINTLESS 2* (*EJ2*/*MADS1*), caused excessively branched inflorescences, probably because of the formation of additional IMs due to delayed FM maturation. Mutations in the *SOC1*-like genes *TOMATO MADS-box gene 3* (*TM3*) and *SISTER OF TM3* (*STM3*) suppress the enhanced branching phenotype of the *j2 ej2* mutant, suggesting an opposite role in FM development (Soyk et al., 2017a; Alonge et al., 2020a).

FM fate is established by FM identity genes that on their turn initiate the expression of floral organ identity genes. In addition to the protein complexes formed by the MADS-domain proteins mentioned above, another complex is crucial for FM specification in tomato. This is a complex encoded by the F-box gene *AN* and its transcription factor partner *FA*. Loss-of-function mutations in *FA* and *AN* block FM maturation, resulting in highly branched inflorescences that do not form flowers, but

are composed of massive vegetative meristems or reproductive meristems (Allen and Sussex, 1996; Molinero-Rosales et al., 1999). *FA* is completely epistatic to *AN*, confirming its key role in specification of floral identity (Allen and Sussex, 1996). Additionally, mutation of a homeobox transcription factor encoded by *COMPOUND INFLORESCENCE* (*S*, homolog of Arabidopsis *WUSCHEL-RELATED HOMEOBOX 9*) delays *AN* expression and thereby FM maturation, allowing additional IM formations, also giving rise to highly branched inflorescences (Quinet et al., 2006b; Lippman et al., 2008a; Park et al., 2012). In contrast, disruption of *TMF*, which causes early flowering by de-repression of *AN*, causes a single-flower inflorescence due to precocious FM maturation (MacAlister et al., 2012b; Huang et al., 2018b).

Plant reproductive shoot architecture

The expression dynamics of the meristem identity genes involved in flowering, shape the plant's architecture. In monopodial species such as Arabidopsis, the IM grows indefinitely after floral transition to develop the inflorescence. In sympodial plants, such as tomato, the meristem is determinate, but due to the formation of flanking IMs, an inflorescence can be formed. Additionally, the plant resumes growth from an axillary meristem (sympodial meristem, SYM), which arises from the axil of the youngest leaf primordium. Plant growth thus continues via the sympodial shoots, where the vegetative and reproductive phases alternate successively to make the complicated tomato plant architecture and enable indeterminate plant growth (Figure 3) (Périlleux et al., 2019).

Despite the differences of monopodial and sympodial systems, the genes that mediate the identity of the SAM are largely the same in flowering plant species. Two homologous proteins from the phosphatidylethanolamine binding protein (PEBP) family, *FT* and *TFL1*, induce the determinate and indeterminate growth of plants, respectively. The FT protein is translated in leaves and then transported via the phloem into the shoot apex to activate flowering. Loss of *FT* function extends the vegetative state, while over-expression of *FT* results in early flowering and a determinate inflorescence (Kardailsky et al., 1999; Kobayashi et al., 1999). Yet, mutations in *TFL1* confer early flowering and result in a determinate inflorescence with a terminal flower (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1997; Ohshima et al., 1997). These antagonistic functions are due to a single amino acid difference in the potential binding pocket of the PEBP family (Hanzawa et al., 2005). Compelling evidence revealed that the *FT/TFL1* balance controls the plant's indeterminate and determinate growth through a dose-dependent relationship. In soybean, the recessive *TFL1* homolog *dt1* conditions the determinate

habit and heterozygotes produce a semi-determinate architecture (Liu et al., 2010). The calibrating functions of *FT*/*TFL1* are highly conserved in many other plant species, including grape (Carmona et al., 2007), sunflower (Blackman et al., 2010), cotton (Si et al., 2018), barley (Comadran et al., 2012) and tomato (Pnueli et al., 1998). Notably, revisiting the dose-dependent roles of *FT*/*TFL1* in shoot architecture suggest opportunities to fine-tune agricultural productivity.

While the role of the *TFL1*-homolog *SP* appears limited in tomato primary shoot- and inflorescence development, *SP* is essential in the tomato sympodial shoot to regulate its transition to reproductive growth. Tomato sympodial shoot compactness is a crucial component for yield, and therefore the *sp* mutation was an important factor during tomato domestication. In the *sp* variety, the indeterminate tomato shoot is transformed into a determinate one, in which the sympodial shoots transition to flowering progressively early and end with an inflorescence (Yeager, 1927). This phenotype is strengthened by the *BL* mutation and suppressed by the *J* mutation (Quinet et al., 2011). *SP* is not expressed in the primary shoot, but in the SYM and other axillary meristems, its mutation induces sympodial shoot flowering, while its overexpression promotes the vegetative phase, leading to an increasing number of leaves between inflorescences (Pnueli et al., 1998; Thouet et al., 2008). Loss of the floral repressor *SP5G* in the *sp* background results in enhanced compact determinate growth (Carmel-Goren et al., 2003; Soyk et al., 2017b) and enabled *de novo* domestication of wild *Solanum pimpinellifolium* (Li et al., 2018; Zsögön et al., 2018). Moreover, the combination of *SP*, as an antiflorigen, with *SFT*, as a florigen, controls tomato shoot architecture in a quantitative and dose-dependent way. *SFT* is completely epistatic over *SP* and *SFT*/*sft* heterozygosity in the *sp* background fine tunes plant size and suppresses determinate growth, ultimately increasing fruit yield compared to both homozygous parents (Shalit et al., 2009; Krieger et al., 2010a; Jiang et al., 2013b; Park et al., 2014). Taken together, maximum tomato yield could be achieved by optimizing the plant architecture by adjusting the *SFT*/*SP* balance.

Crop improvement by modification of the flowering network

The timing of flowering is of great importance for crop production. In vegetative crops, such as cabbage and sugar beet, early bolting and flowering decreases the vegetative yield, whereas for species with a reproductive product it is just the opposite. Modifications of flowering genes can be beneficial for crop improvement and the flowering time trait has therefore been a target of domestication and selective breeding. The molecular principles and pathways of flowering time have been extensively studied in model species, which facilitates the understanding of the

flowering pathway in major crops, due to the functional conservation of the key regulators. In particular, the vernalization and photoperiod pathways play key roles in the adaptation of crops to different climates and geographical areas.

One of the numerous examples of the selection of beneficial QTLs and genes in the flowering pathway is the allelic variation fixed in tomato for the genes *SP5G* and *FTL1*, where cultivars have reduced long-day and short-day flowering responses, allowing them to spread globally (Soyk et al., 2017b; Song et al., 2020). With the development of the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR associated proteins) system, the manipulation of flowering genes for agricultural applications comes in sight, allowing a much more rapid crop improvement (at least in countries where the GMO legislation approves CRISPR/Cas-generated crops). CRISPR/Cas has recently been rapidly further developed and widely used for genome editing in many crop species as it can be easily programmed and applied at minimal costs. Since the first application in plants about 10 years ago (Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013), the CRISPR technology has had a major impact on crop improvement, owing to its capability of precise manipulation of plant genomes. It has also been a revolution in the fundamental research, allowing a straightforward generation of knock-out mutants and thereby rapid functional characterization of genes. So far, flowering time in the major crops, such as maize (Huang et al., 2018a), rice (Brambilla et al., 2017), tomato (Li et al., 2018), and soybean (Cai et al., 2018), have been studied with CRISPR mutagenesis to modulate flowering responses.

However, crop improvement via CRISPR gene editing can only be efficient if there is enough knowledge on the gene regulatory networks that regulate flowering. Although progress has been achieved in understanding crop flowering time, only the key regulators are identified. To fine tune flowering in crop species by gene editing, detailed regulatory networks need to be available, including not only the key genes, but also their mutual interactions, upstream regulators and downstream targets.

Outline of this thesis

Tomato is economically an important vegetable crop, and its fruit yield is among others determined by the timing of flowering, inflorescence architecture and shoot determinacy. These traits are biological consequences of the activities of reproductive meristems, which are regulated by endogenous conditions, phytohormones and flowering pathway genes. The *FUL* gene, encoding a pleiotropic MADS domain transcription factor in Arabidopsis, has been implicated in different flowering pathways. Its homologs play conserved roles in flowering and

inflorescence development in many other species but had not yet been thoroughly investigated in tomato. The tomato genome carries four *FUL*-like genes (*SlFULs*: *FUL1*, *FUL2*, *MBP10*, *MBP20*), among which only *FUL1* and *FUL2* are known to be involved in regulation of fruit development. The aim of the studies described in this thesis is to investigate the functions of the *SlFUL* genes in flowering and inflorescence development, and their redundancy/interaction with *MC* and *SP*, as well as their regulation of downstream target genes, such as the *SlCKXs*. To do so, we employed CRISPR mutagenesis, transcriptome analysis, protein-protein interaction analysis, gene expression analysis, and investigated meristem development in detail.

Chapter 2 shows that *FUL2* and *MBP20* promote the floral transition and repress inflorescence branching by inducing floral meristem maturation. *FUL1* fulfils a less prominent role and appears to depend on FUL2 and MBP20 for its upregulation in FM/IM, and *MBP10* has probably lost its function. The tomato FUL-like proteins cannot homodimerize with each other and potentially form distinct complexes in the TM and FM. Transcriptome analysis of the primary shoot meristems identified four negative repressors of cytokinin signalling that are upregulated during the floral transition in *ful1 ful2 mbp10 mbp20* mutants. FUL2 and MBP20 can bind *in vitro* to the upstream regions of these genes, and loss of *SlCKX6* caused early flowering, suggesting that SlFULs can probably directly stimulate cell division in the meristem upon the transition to flowering. The control of inflorescence branching may be regulated by repression of transcription factors such as *TM3* and *APETALA 2b* (*AP2b*).

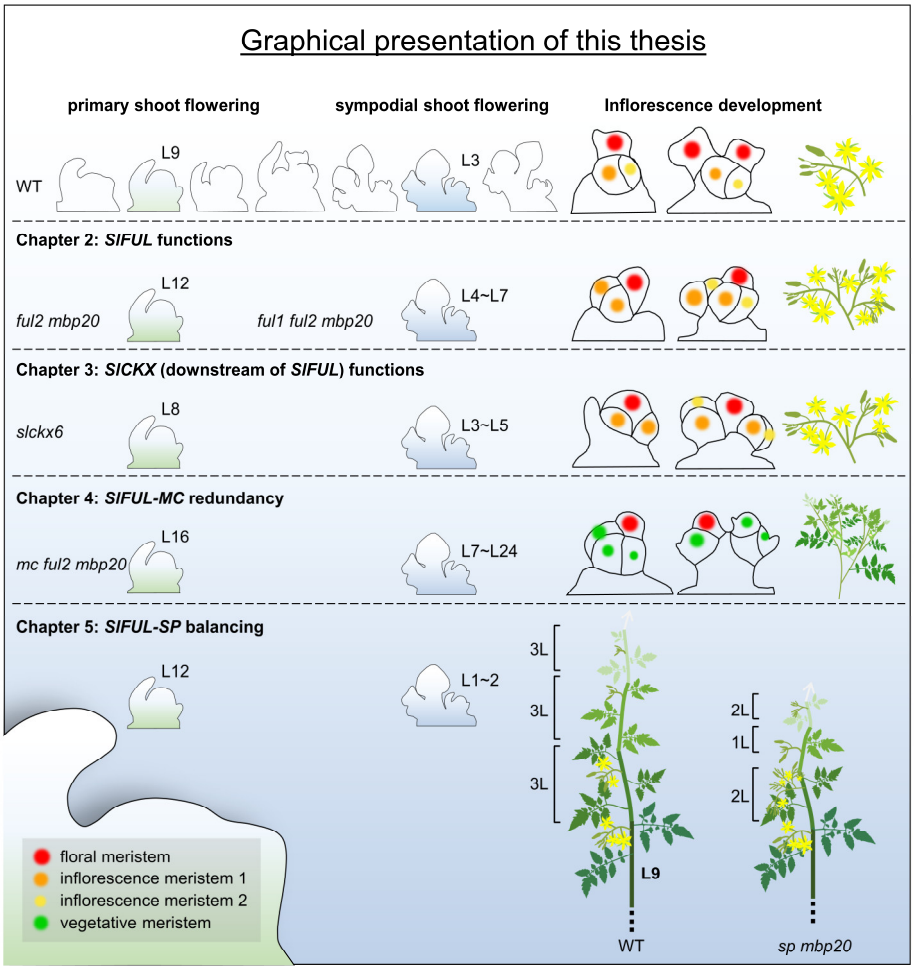
The results in **Chapter 3** indicate that *SlCKXs* play a prominent role in reproductive meristem development in tomato. CRISPR mutants of *SlCKX5*, *SlCKX6*, and *SlCKX8* revealed interesting phenotypes in both flowering time and inflorescence branching, where *SlCKX6* alone appeared to regulate primary shoot flowering, while all three genes were involved in the control of sympodial shoot flowering time and the regulation of inflorescence branching by simultaneous appearance of two IMs on both flanks of the FM.

Chapter 4 reports that combined mutations of *FUL2*, *MBP20*, and *MC* caused somewhat enhanced late primary shoot flowering, whereas the sympodial shoot flowering was massively delayed, leading to a severely disperse shoot structure. In addition, the delayed FM maturation in the *mc ful2 mbp20* triple mutant leads to additional formation of flanking meristems, which adopt a vegetative fate instead of IM fate, resulting in a massively bushy inflorescence with limited productivity. We

demonstrate that *FUL2/MBP20* play a major role in floral transition and FM determinacy while *MC* is most important for specifying IM identity.

Chapter 5 describes a modification of shoot architecture by simultaneous manipulation of the flowering promoter *MBP20* and the repressor *SP*. Loss of *MBP20* restores the indeterminate growth of *sp* mutants with accelerated sympodial cycling, resulting in an enhanced inflorescence complexity and compactness. The *sp mbp20* mutant plants produce more flowers and have increased fruit yield per inflorescence compared to wild-type plants, without any negative effects on fruit development.

Chapter 6 summarized and discusses the major results in this thesis and highlights possible directions for further research.



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CHAPTER 2

FRUITFULL-like genes regulate flowering time and inflorescence architecture in tomato

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ABSTRACT

The timing of flowering and the inflorescence architecture are critical for the reproductive success of tomato (*Solanum lycopersicum*), but the gene regulatory networks underlying these traits have not been fully explored. Here we show that the tomato *FRUITFULL*-like (*FUL*-like) genes *FUL2* and *MADS-BOX PROTEIN 20* (*MBP20*) promote the vegetative-to-reproductive transition and repress inflorescence branching by inducing floral meristem maturation. *FUL1* fulfils a less prominent role and appears to depend on FUL2 and MBP20 for its upregulation in the inflorescence- and floral meristems. *MBP10*, the fourth tomato *FUL*-like gene, has probably lost its function. The tomato FUL-like proteins cannot homodimerize in *in vitro* assays, but heterodimerize with various other MADS-domain proteins, potentially forming distinct complexes in the transition meristem and floral meristem. Transcriptome analysis of the primary shoot meristems revealed various interesting downstream targets, including four repressors of cytokinin signalling that are upregulated during the floral transition in *ful1 ful2 mbp10 mbp20* mutants. FUL2 and MBP20 can also bind *in vitro* to the upstream regions of these genes, thereby probably directly stimulating cell division in the meristem upon the transition to flowering. The control of inflorescence branching does not occur via the cytokinin oxidase/dehydrogenases (CKXs) but may be regulated by repression of transcription factors such as *TOMATO MADS-box gene 3* (*TM3*) and *APETALA 2b* (*AP2b*).

INTRODUCTION

The MADS-box transcription factor gene family is involved in almost every developmental process in plants (Smaczniak et al. 2012a), and the members of the angiosperm-specific *APETALA1/FRUITFULL* (*AP1*/*FUL*) subfamily play key roles in flowering and fruit development (Litt and Irish 2003; McCarthy et al. 2015). In the core eudicots, the *AP1/FUL* subfamily consists of three clades, *euAP1*, *euFULI* and *euFULII* (Litt and Irish 2003). The *Arabidopsis thaliana* genome carries four *AP1/FUL*-clade genes, with *AP1* functioning as a key regulator of floral initiation and floral meristem (FM) establishment, and acting as an A-class gene in the ABC model (Ferrándiz et al. 2000; Kaufmann et al. 2010), promoting perianth identity (Mandel et al. 1992; Theissen and Saedler 2001). Its lower-expressed paralog *CAULIFLOWER* (*CAL*) functions to a large extent redundantly with *AP1* (Bowman et al. 1993; Ye et al. 2016). The *euFULII*-clade gene *AGAMOUS-LIKE 79* (*AGL79*) appears to have a minor function in roots (Gao et al. 2018), whereas the *euFULI* gene *FUL* is a pleiotropic gene. In addition to its key role in fruit development (Gu et al. 1998), *FUL* regulates many aspects of flowering in Arabidopsis, including flowering time

(Ferrándiz et al. 2000; Melzer et al. 2008), repression of inflorescence meristem (IM) identity (together with *AP1/CAL*; Ferrándiz et al., 2000), inflorescence architecture (Bemer et al. 2017), axillary inflorescence outgrowth (together with *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (Karami et al. 2020)) and IM termination (Balanzà et al. 2018). In a wide range of angiosperm species, *FUL*-like genes regulate flowering (Ferrándiz et al. 2000; Jia et al. 2015; Jaudal et al. 2015; Berbel et al. 2012; Li et al. 2019; Pabón-Mora et al. 2012; Ping et al. 2014; Zhang et al. 2021) and fruit development (Bemer et al. 2012; Gu et al. 1998; Jaakola et al. 2010; Pabón‐Mora et al. 2013; Zhao et al. 2019). In tomato (*Solanum lycopersicum*), the *euFULI*-clade genes *S. lycopersicum FUL1* (*SlFUL1*/*TM4*/*TDR4*, hereafter called *FUL1*) and *SlFUL2* (*FUL2*/*MBP7*, hereafter called *FUL2*) play important roles in fruit development and ripening (Shima et al. 2013; Bemer et al. 2012; Wang et al. 2019), but flowering phenotypes have not yet been described for these genes, nor for the tomato *euFULII*-clade genes *MADS-BOX PROTEIN 10* (*MBP10*) and *MBP20*. This is remarkable given the strong upregulation of *FUL1*, *FUL2* and *MBP20* expression during the transition from shoot apical meristem (SAM) to FM/IM (Park et al. 2012).

Flowering is an important agricultural trait in tomato, because the onset and termination of flowering, as well as the inflorescence architecture, determine crop yield. Tomato is also an interesting model species considering its sympodial shoot architecture, which is distinct from that of the monopodial Arabidopsis. While the Arabidopsis SAM develops into an IM, which subsequently forms FMs on its flank (indeterminate inflorescence), the tomato SAM domes to form the transition meristem (TM) that terminates directly into an FM (determinate inflorescence), but forms a new IM on its flank. This iterative process results in a zigzagged inflorescence (Lippman et al. 2008). Moreover, tomato has a compound shoot, which resumes vegetative growth from the axillary meristem of the youngest leaf axil when the inflorescence has formed. After activation of the axillary meristem (then called sympodial meristem, SYM), the shoot forms three leaves before terminating again into the first flower of the second inflorescence (Lippman et al. 2008; Pnueli et al. 1998; Szymkowiak and Irish 2006), upon which a new axillary meristem takes over vegetative growth. This process forms the compound shoot, where three leaves and an inflorescence comprise a sympodial unit, a pattern that is endlessly repeated in the wild-type (WT) tomato.

The genes that regulate these flowering processes have been very well studied in the indeterminate Arabidopsis inflorescence, where the floral integrators FLOWERING LOCUS T (FT) and SOC1 regulate the transition to flowering, after which TERMINAL FLOWER 1 (TFL1) determines IM fate by repressing FM genes

such as *LEAFY* (*LFY*) and *AP1* (Zhu et al. 2020; Sablowski 2007; Lee and Lee 2010; Serrano-Mislata et al. 2017), while AP1 on its turn represses *TFL1*, so that clear borders between the FM and IM are achieved (Liu et al. 2013; Goslin et al. 2017). The variation in inflorescence structures of different species can be largely explained by different temporal and spatial expression of flower-repressing *TFL1* homologs and flower-inducing *LFY/AP1/FUL* homologs on the other side (Périlleux et al. 2019; McGarry and Ayre 2012). In legumes, for example, the indeterminate inflorescence does not form FMs on its flank, but secondary IMs, due to repression of the *TFL1*-homolog in these meristems by the euFULII clade proteins VEG1 (pea, *Pisum sativum*) or MtFUL1-c (Medicago, *Medicago truncatula*) (Cheng et al. 2018; Zhang et al. 2021; Berbel et al. 2012). These secondary IMs do form FMs, resulting in a compound inflorescence (Benlloch et al. 2015).

In tomato, *SINGLE FLOWER TRUSS* (*SFT*, homolog of *FT*), *FALSIFLORA* (*FA*, homolog of *LFY*) and MACROCALYX (*MC*, homolog of *AP1*) are essential for the transition to flowering and control of FM identity similar to their orthologs in Arabidopsis (Molinero-Rosales et al. 1999; Molinero-Rosales et al. 2004; Yuste-Lisbona et al. 2016). However, mutants in the *TFL1*-ortholog *SELF-PRUNING* (*SP*), which is not expressed in the primary vegetative SAM (VM), but highly expressed in axillary meristems (Thouet et al. 2008), lose this growth indeterminacy and terminate their SYMs early, resulting in termination of growth after a few sympodial units (Pnueli et al. 1998). Inflorescence architecture is normal in an *sp* mutant (Pnueli et al., 1998). Instead, tomato inflorescence architecture is influenced by other factors that regulate the timing of FM maturation (Lippman et al. 2008). Failure of meristem maturation in the *fa* and *anantha* (*an*, *homolog of UFO*) mutants, or largely delayed maturation in *compound inflorescences* (*s*, homolog of *WOX9*) mutant induce additional IM formations, resulting in highly branched (compound) inflorescences (Park et al. 2012; Soyk et al. 2017; Souer et al. 2008; Szymkowiak and Irish 2006; Chae et al. 2008; Lippman et al. 2008). Conversely, precocious activation of the AN/FA complex leads to early FM maturation and thus early flowering (MacAlister et al. 2012). In addition to these factors, several MADS-domain transcription factors function in tomato flowering, mainly in conferring IM or FM identity. In the *jointless* (*j*, homolog of *SVP*) and *mc* mutants, flowering is delayed and the inflorescence reverts to vegetative growth after a few flowers, probably because the FM flanking meristems adopt VM/SYM identity instead of IM fate (Yuste-Lisbona et al. 2016; Szymkowiak and Irish 2006). Mutations in the *SEPALLATA* (*SEP*)-like genes *JOINTLESS 2* (*J2/SlMBP21*), *ENHANCER OF JOINTLESS 2* (*EJ2/MADS1*) and the *SOC1*-like genes *TM3* and *SISTER OF TM3* (*STM3*) affect inflorescence branching through yet uncharacterized mechanisms (Roldan et al. 2017; Soyk et al. 2017; Soyk

et al. 2019; Alonge et al. 2020). Interestingly, the *tm3 stm3* mutations suppress the enhanced branching phenotype of the *j2 ej2* mutant (Alonge et al. 2020), suggesting that these MADS-domain transcription factors have an opposite function in FM development. Natural mutations or structural variants in several of these MADS-box genes have been important for domestication, either through the regulation of flower/fruit abscission (*MC*, *J*, *J2*) or inflorescence architecture (*J2*, *EJ2*, *TM3*, *STM3*) (Soyk et al. 2019; Nakano et al. 2012; Liu et al. 2014; Alonge et al. 2020). Thus, although tomato inflorescence development differs fundamentally from that of monopodial species such as Arabidopsis, the orthologs of several important Arabidopsis flowering and IM genes are also essential in tomato. However, it is yet unclear if and how the *FUL*-like genes (*SlFULs*) function in the tomato flowering regulatory network. Here, we investigated the developmental roles of the four *SlFULs* in tomato by CRISPR/Cas9-mutagenesis and transcriptome profiling. We demonstrate that sub-functionalization has occurred after duplication within the Solanaceae *euFULI* and *euFULII* clades, and that the *FUL1* sequence has undergone further divergence during tomato domestication and breeding. *FUL2* and *MBP20* are highly expressed in the meristem during the vegetative-to-reproductive transition to additively promote tomato flowering and to repress inflorescence branching together with *FUL1*. Transcriptome analysis in the *ful1 ful2 mbp10 mbp20* quadruple mutant revealed that the *SlFULs* act probably parallel to, or downstream of, previously described key regulators such as *SFT*, *FA* and *AN* during both the VM-to-TM transition and the establishment of inflorescence architecture. Furthermore, our target gene analysis revealed that the delay in transition to flowering may be explained by reduced cytokinin (CK) signalling as a result of upregulation of cytokinin oxidases/dehydrogenases (*CKXs*), while the increased branching is caused by delayed FM maturation as a possible result of specific MADS-domain and *AP2*-like transcription factors that are upregulated in the mutant.

RESULTS

Expression patterns and protein–protein interaction profiles differ between the SlFULs

To investigate to what extent the SlFULs may have overlapping functions in specific organs, we performed expression profiling using reverse transcription-quantitative PCR (RT-qPCR) in the cultivar Moneyberg (Figure 1A). *FUL1* and *FUL2* were expressed very weakly during vegetative growth and had increased expression in the IM-enriched sample. Their expression remained high throughout reproductive development, where both genes showed considerable expression in all floral whorls

and all stages of fruit development. *FUL2*, in particular, was strongly expressed in all floral organs, early fruits and ripening fruits, while *FUL1* expression was moderate until the fruit ripening phase, when it increased strongly as reported previously (Bemer et al. 2012). Our data also revealed striking differences in spatial expression, with *MBP10* and *MBP20* expressed to much lower levels than *FUL1* and *FUL2* in the reproductive tissues, except for the expression of *MBP20* in the IM. *MBP10* especially was extremely weakly expressed, with detectable levels in stem and flower bud only.

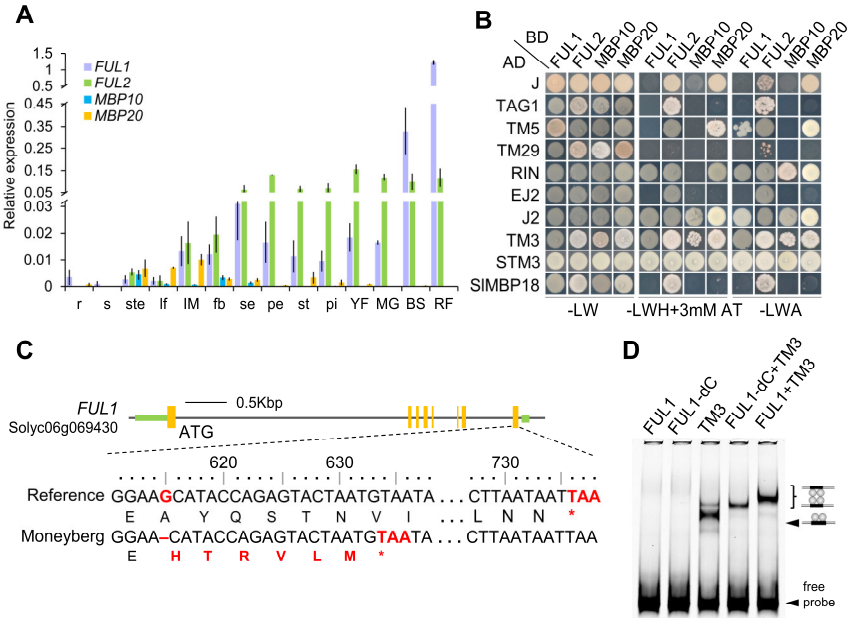

Figure 1. **Characterization of the tomato *FUL*-like genes**. **(A)** Relative expression profiles of *SlFUL* genes in different organs obtained by qRT-PCR. r: two-week-old root; s: two-week-old shoot; st: stem below apex; lf: young leaf; IM: inflorescence meristem (dissected apex); bud: closed flower bud; se: sepal; pe: petal; st: stamen; pi: pistil; YF: young fruit; MG: mature green fruit; BR: breaker stage fruit; RR: red ripe fruit. The error bars indicate ±SD based on three biological replicates. **(B)** Yeast two-hybrid assays showing the protein interactions of the FUL-like proteins with other tomato MADS-domain proteins. L, leucine; W, tryptophan; H, histidine; A, adenine; 3-AT, 3-amino-1,2,4-triazole. **(C)** The location of the one-nucleotide deletion present at the 3' end of the *FUL1* gene in the cultivar Moneyberg, resulting in a protein lacking the C-terminus. Orange rectangle indicates exons. **(D)** EMSA assays showing that the FUL1 truncated protein (FUL1-ΔC) is functional *in vitro*.

MADS-domain transcription factors regulate multiple developmental processes by forming dimeric or higher-order complexes (De Folter et al. 2005; Immink et al. 2009). To investigate which protein complexes may be formed by the different SlFUL

proteins, we performed a yeast-two hybrid (Y2H) assay to identify the interactions of FUL1, FUL2, MBP10 and MBP20 with other tomato MADS-domain family proteins (Figure 1B, Supplemental Figure S1). We chose a set of proteins homologous to MADS-domain proteins known to interact with Arabidopsis FUL (AtFUL) (De Folter et al. 2005). While AtFUL can form homodimers (Smaczniak et al. 2012b), none of the SlFUL proteins possessed this capacity, nor could they heterodimerize with each other (Supplemental Figure S1). Of the tested MADS-domain proteins, MC, SlMBP9, SlMBP12, SlMBP13, SlMBP14, SlMBP22 and SlMBP24 did not interact with any of the SlFUL proteins. Our screen showed that the SlFULs could interact with ten other tomato MADS-domain proteins, and we observed clear differences between the interaction profiles of FUL1, FUL2, MBP10 and MBP20. All SlFUL proteins interacted with J, J2, TM3, STM3 and the fruit ripening regulator MADS-RIN. FUL2 exhibited the most extensive interaction network, interacting with all ten proteins. It is the only protein that strongly interacted with the SEP-like proteins EJ2 and LeSEP1/TM29 (TM29-BD only with FUL2-AD, see Supplemental Figure S1), and with TAG1 (ortholog of AG) (Figure 1B and Supplemental Figure S1). The latter interaction is especially interesting in the light of the high expression of *FUL2* in pistils, where FUL2 may have a specific function in a complex with the co-expressed TAG1. In addition to these specific interactions, FUL2, FUL1 and MBP20 share interactions with the SEP-like protein TM5 and with FOREVER YOUNG FLOWER-LIKE (FYFL/SlMBP18, homolog of AGL42), and FUL1 could also weakly interact with EJ2 (Supplemental Figure S1). Some of the interaction pairs we tested have been investigated before in a tomato MADS-domain interaction screen (Leseberg et al. 2008). We could reproduce all the previous results, except for interactions of FUL2 and MBP20 with SlMBP13, and FUL2 with SlMBP24. In conclusion, FUL2 can form most protein–protein interactions, which, together with its broad expression pattern, suggests that it can fulfil multiple functions in tomato, similar to AtFUL. FUL1 and MBP20 share a reduced set of interaction partners and MBP10 has the smallest set of interactors. The low number of interactors for MBP10 in combination with its weak overall expression pattern hints at relaxed selective pressure on this gene.

Different variants of the *FUL1* gene exist in tomato cultivars

Upon cloning and sequencing of the *FUL1* cDNA of tomato cv. Moneyberg, which was used in our experiments, we noticed a deletion of the 5th base (G) of the last exon as compared to the reference sequence (cv. Heinz). This deletion is predicted to result in a 205 amino acid protein, lacking the C-terminal 40 amino acids as compared to the reference (Figure 1C). Inspection of the genome sequence of 38 re-sequenced cultivated tomato varieties (Consortium et al. 2014) showed that

approximately half (17) contain this deletion (Supplemental Figure S2A). The latter included the much-studied cv. 'Ailsa Craig'. The deletion was not detected in any of the re-sequenced wild accessions, suggesting that it may have first emerged after domestication. Although the deletion results in a C-terminally truncated protein (FUL1-ΔC), no differences in interactions were observed in a Y2H assay (Supplemental Figure S2B). An Electrophoretic Mobility Shift Assay (EMSA) also revealed that FUL1-ΔC can bind to a CArG-box-containing DNA fragment as a heterotetramer with TM3 (Figure 1D). Thus, *in vitro* DNA-binding and protein–protein interaction capacities appear normal for FUL1-ΔC, indicating, together with the already described fruit ripening function for FUL1-ΔC in the cultivars Moneyberg and Ailsa Craig (Wang et al., 2019; Bemer et al., 2012), that the truncated protein is functional. To investigate whether there could be a link between the occurrence of the *FUL1-ΔC* allele and certain crop traits, we examined the recently published dataset of Roohanitaziani et al. (2020), in which a wide variety of cultivars and wild species has been characterized for many traits, including flower/fruit abscission, flowering time, inflorescence architecture, fruit development and fruit ripening. However, we did not find significant differences in any of these traits between the cultivars with a full-length *FUL1* allele and those with a truncated allele (Supplemental Figures S2C and S2D), suggesting that the occurrence of the *FUL1-ΔC* allele does not have a major influence on the investigated features.

FUL2 and MBP20 promote flowering in the primary and sympodial shoots

To dissect the biological roles of the *SlFUL* genes *in planta*, we generated loss-of-function single- and higher-order mutants with the CRISPR/Cas9 method. The first coding exon of each gene was targeted with three single-guide RNAs. After stable tomato transformation of the cultivar Moneyberg, we screened several independent first-generation (T0) transgenic lines for the presence of insertion/deletion (indel) alleles by PCR and sequencing. Then we generated the progeny of the primary transgenics (T1) and selected two different homozygous indel alleles for each gene, encoding truncated proteins caused by frameshifts and premature stop codons (Supplemental Figure S3). T2 phenotyping of the selected mutants revealed that the plants with homozygous knock-out alleles for either *FUL2* or *MBP20* exhibited delays in primary shoot flowering, switching to reproductive growth after 13 leaves, compared to 11 in the WT (Figure 2A). The number of days to first flowering was also significantly increased but was more variable between individuals of the same genotype (Supplemental Figure S4A). No significant effect in the number of leaves was observed for the *ful1* or *mbp10* single mutants (Figure 2A, Supplemental Figure S4B). The observed delay in the transition to flowering was more pronounced in

higher-order mutants, with approximately three leaves extra in *ful2 mbp20*, *ful1 ful2 mbp20* and *ful1 ful2 mbp10 mbp20* compared to the WT (Figure 2A). The fact that neither the *ful1* nor the *mbp10* mutations enhanced the mutant phenotype, suggests that *FUL2* and *MBP20* are the most important *FUL*-like genes for promoting the floral transition. In addition to a delay in primary shoot transition, we also observed late flowering in the sympodial shoots of the same set of mutants, increasing to an average of four leaves per sympodial shoot, while the WT always has three (Figures 2B and 2C, Supplemental Figures S4C and S4D). To further investigate the delayed flowering phenotype, we imaged the primary shoot meristems of the WT and quadruple mutant at different leaf stages and determined their size, as well as the timing of the reproductive stage transition (Figure 2D and Supplemental Figures S4E and S4F). We did not observe differences in the size or shape of the meristems in the vegetative stage but discovered that the timing of meristem doming differed. In the WT, meristem transition proceeded rapidly with a visible doming after formation of nine leaves. However, in the quadruple mutant, doming was initiated later and proceeded slower, resulting in development of the FM only after 12 leaves. In conclusion, *FUL2* and *MBP20* additively regulate the timing of flowering in both the primary shoot and the sympodial shoots. Later during development, *ful1* single mutants also showed an increase in leaf number (Figure 2B, Supplemental Figure S4D), suggesting that *FUL1* plays a minor role as well.

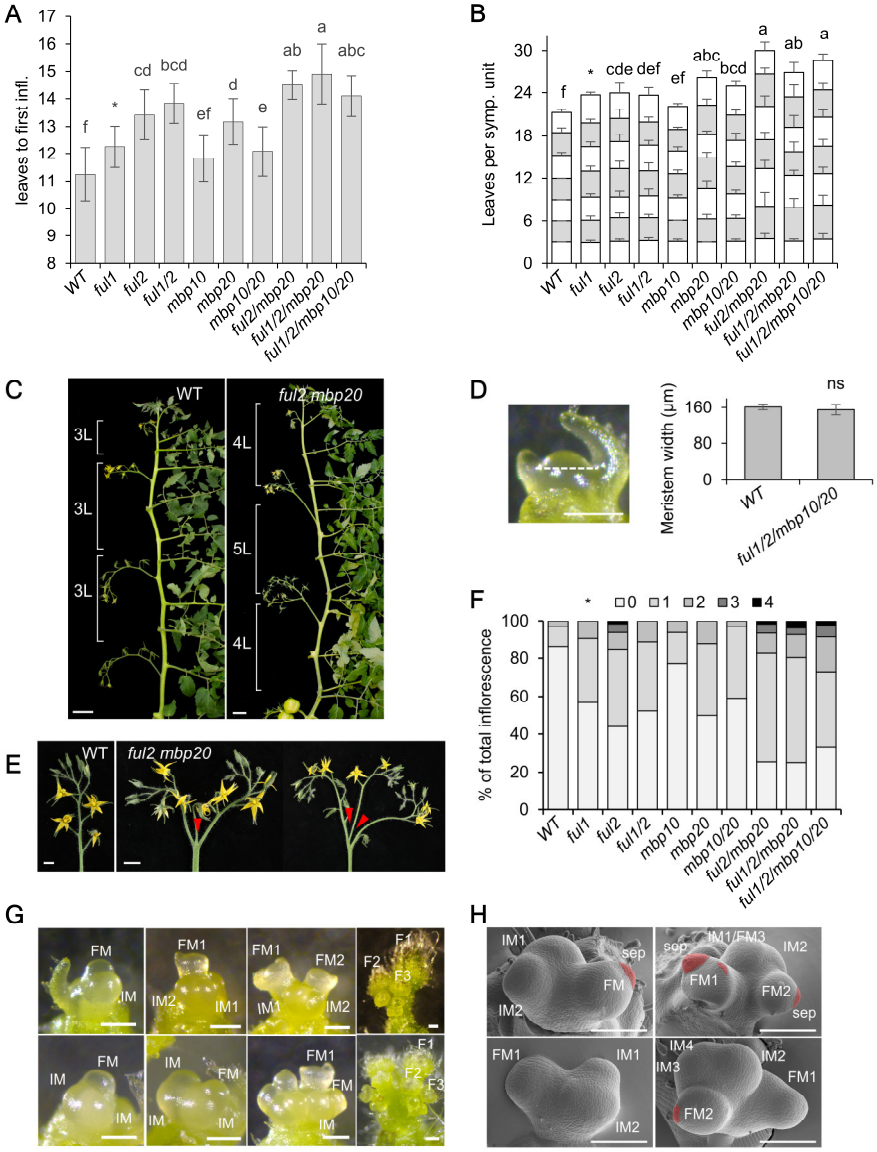

Figure 2. Disruption of the *FUL*-like genes results in delayed flowering and enhanced inflorescence branching. (A) Quantification of primary shoot flowering for wild-type (WT) and *slful* mutants. **(B)** Number of leaves per sympodial unit for the first seven units in WT and *slful* mutants. The average of the leaves per unit was used to test the significance. **(C)** Representative sympodial shoots from WT plants and the *ful2 mbp20* mutant. L: leaf; Scale bars: 5 cm. **(D)** Quantification of SAM width from WT and *quadruple* mutant plants. VMs were in the 10-leaf stage. The dashed line marks the width for measurement. ns: not significant. White bar: 200 µm. **(E)** Representative images of wild-type and mutant (branched) inflorescences. Red arrowheads indicate branching events. Scale bars: 2 cm. **(F)** Proportion

of branched inflorescences per branching category for the indicated genotypes. The numbers (1 to 4) indicate the number of branching events. **(G)** and **(H)** Developmental series of sympodial meristems of WT and quadruple mutant imaged by stereomicroscope (G) and by scanning electron microscope (H). In G and H, the upper panels are from WT and the lower from quadruple mutants. In H, developing sepal primordia are marked in red. FM: floral meristem; IM: inflorescence meristem; F: flower; White bar: 200 µm. In A and C, mean values (± SD) were compared between genotypes using one-way ANOVA followed by a post hoc LSD test, different letters indicate the difference at $P<0.05$ level, six individual T2 offspring plants were analysed per line and the data from the two different genotypes were combined for each mutant (e.g. 2x6 individuals for *ful1* etc.). The asterisk in A,B and F indicates that the *ful1* data is acquired from a second phenotyping experiment. ns: no significance.

FUL1, FUL2 and MBP20 control inflorescence architecture

We observed that the mutant plants had more branched inflorescences than WT plants, which typically produced only non-branched inflorescences (Figure 2E). We quantified the branching events for the first seven inflorescences of each plant in the T2 generation. Except for *mbp10*, all mutants showed increased inflorescence complexity, ranging from bi-parous to quintuple-parous inflorescences (Figure 2F). Notably, 13.6% of the inflorescences from WT plants branched, while *ful1*, *ful2* and *mbp20* lines produced 43.1%, 56.1% and 50% branched inflorescences, respectively. In higher-order mutants, branching increased further to ~75% in both the *ful2 mbp20* and the *ful1 ful2 mbp20* mutants. *mbp10* mutants were hardly branching, similar to the WT, while *mbp10 mbp20* mutants were identical to *mbp20* mutants. These results indicate no additional contribution of *mbp10* or *ful1* to the branching phenotype of the mutants. Surprisingly, the *ful1* mutant did exhibit enhanced branching, but its mutation did not further enhance the phenotype of the *ful2 mbp20* mutant, suggesting that *FUL1* function depends on *FUL2* and/or *MBP20* (Supplemental Figure S4G). Interestingly, all first appearing inflorescences did not branch, except for the first inflorescence of *ful2 mbp20* (3 plants out of 11) and *ful1 ful2 mbp20* (2 plants out of 11). Higher order branching events (i.e. quintuple parous) were observed only in mutant lines where *ful2* was included, suggesting that *FUL2* has the most prominent role in the repression of inflorescence branching. We quantified the number of flowers on the second to fourth inflorescences. The more complex inflorescences developed more flowers, increasing from on average 10 in WT inflorescences to approximately 20 in the higher order mutants (Supplemental Figure S4H). To investigate whether the increased branching could also be linked to delayed FM maturation, as observed for the *j2 ej2* (Soyk et al. 2017), *s* and *an* mutants (Lippmann et al., 2008), we examined different stages of meristem development under the microscope (Figure 2G). We observed delayed FM

development of the sympodial shoot meristems, which initiated sepal primordia slower than the WT, allowing the formation of a second IM before FM maturation. We checked these observations with SEM pictures (Figure 2H), which confirmed that meristem shape does not differ between WT and quadruple mutant, but that the FMs of the mutant develop much slower. This is clearly visible in Figure 2H, where the WT has already two FMs with initiated sepal primordia, while mutant FM development is delayed, allowing the formation of an additional IM, which is the earliest indication of branching. The frequency of additional IMs was variable, but most similar to that of the *j2 ej2* mutant (Soyk et al. 2017). In conclusion, mutations in *FUL2* and *MBP20*, individually or combined, result in increased branching during inflorescence development. Both genes thus regulate inflorescence architecture in an additive manner, probably by regulating FM maturation. *FUL1* is also involved in this process as is shown by the *ful1* single mutant phenotype, but its role is masked in higher order mutants that contain *ful2* and *mbp20* alleles.

MBP10 and *MBP20* do not contribute to fruit development and ripening

FUL1 and *FUL2* were reported as redundant regulators of tomato fruit ripening, and *FUL2* has an additional function in early fruit development (Wang et al. 2019; Bemer et al. 2012). Since *MBP20* is weakly expressed in carpels and early stages of fruit development (Figure 1A), we wondered whether it could function in fruit development as well. We therefore examined fruit development and ripening in the different mutant lines. As reported previously, the *ful2* mutant fruits were smaller with stripes on the pericarp, while the *ful1 ful2* mutant fruits were more severely impaired in ripening (Wang et al. 2019). *ful2 mbp20* mutant fruits had the same phenotype as *ful2* fruits, while triple (*ful1 ful2 mbp20*) and quadruple fruits resembled *ful1 ful2* fruits in terms of width, Brix value, number of locules, pericarp stripes and overall external and internal appearance, indicating that *MBP10* and *MBP20* do not contribute to fruit development and ripening (Supplemental Figures S5 and S6). Remarkable was the high Brix values of fruits that contained *ful2* mutant alleles, although this may to a large extent be due to the smaller size of *ful2* fruits (Supplemental Figures S5A and S5B). Interestingly, the number of locules was slightly, but significantly, enhanced in *ful2* single mutants, and in most mutant combinations that contained *ful2* (Supplemental Figures S5C and S5D), suggesting that *FUL2* could have an additional role in the regulation of FM termination.

Dynamic expression of MADS-box genes in the meristem

The CRISPR mutant analysis revealed that *FUL2* and *MBP20* promote the transition from vegetative to reproductive development and control inflorescence architecture. To further unveil the role of the *SlFULs* in flowering, we conducted RNA-seq to compare the transcriptome dynamics during three consecutive stages (VM, TM, FM) of meristem development between the WT and quadruple mutant. For each stage, over 30 meristems from a batch of plants were dissected and pooled for RNA extraction. Three independent batches were grown in the greenhouse at different time points to serve as biological replicates. For practical reasons, the FM and flanking IM were harvested together (further referred to as FIM) (see Figure 3A). High-throughput sequencing yielded a minimum of 30M reads per sample. A PCA plot was generated of all 18 samples, which showed clear separation of the VM, TM and FIM samples, although there was quite some variation between the individual TM samples, probably reflecting the transient nature of this stage (Supplemental Figure S7A). We first determined the expression of the *SlFULs* in the different stages of meristem development, revealing dynamic expression changes through the vegetative-to-reproductive transition for *FUL1*, *FUL2* and *MBP20* (Figure 3B). *FUL2* and *MBP20* are already expressed at the VM stage, but their expression highly increased in the TM stage. *FUL1* is more weakly expressed in the VM, but also reaches high expression levels in the TM and FIM. The higher expression of *FUL2* and *MBP20* in the VM stage is in line with their prominent role in the determination of flowering time. Of the MADS-box genes encoding SlFUL interactors, *J* was highly expressed in all three stages, while the expression of the *SEP*-like genes *EJ2*, *TM29* and *J2* gradually increased from practically absent in VM to clearly expressed in FIM. The *SOC1*-homologs *TM3* and *STM3* were also expressed in all three stages (Figure 3C), but their expression decreased in the FIM in contrast to that of *EJ2*, *TM29* and *J2*. The other potential SlFUL interactors were only weakly expressed. To validate the RNA-seq data analysis, we confirmed the expression patterns of the *SlFULs*, and the genes encoding putative interactors, with RT-qPCR analysis on pooled meristem samples from independently grown batches (Supplemental Figures S7C and S7D).

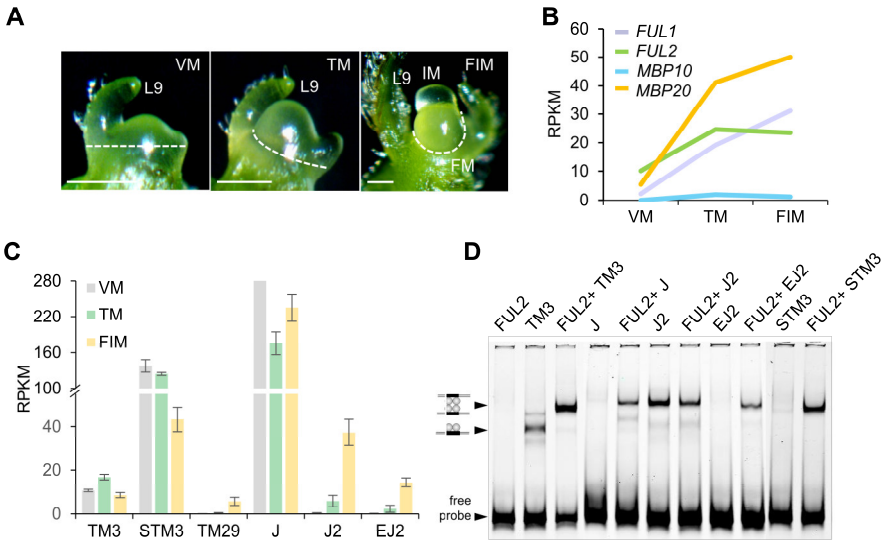

Figure 3. **Gene expression dynamics in the primary shoot meristem. (A)** Manual microdissection of the three successive meristem stages of primary shoot meristems for transcriptome profiling. Dashed lines indicate the dissected tissues. White bar: 100 µm. **(B)**, **(C)** Normalized gene expression (RPKM) of the *FUL*-like genes and *TM3, STM3*, *TM29*, *J, J2*, and *EJ2* in wild-type meristem stages. The values shown (mean ± SD) are the average of three replicates. **(D)** EMSA assays showing FUL2 interactions with MADS-domain proteins. VM: vegetative meristem; TM: transition meristem; FM: floral meristem. IM: inflorescence meristem; FIM: FM and IM.

The combination of the expression and interaction data provides insight into the MADS-domain complexes that may act *in planta*. *J2* and *EJ2* are hardly expressed in the VM/TM, but considerable expression was detected in the FIM samples which is probably the result of high FM expression, as detected by Park et al. (2012) (Thouet et al. 2012; Park et al. 2012; Alonge et al. 2020). Thus, FUL1, FUL2 and MBP20 probably form a complex with J2 and EJ2 in the FM to promote FM maturation. This is in agreement with the enhanced branching phenotype in *j2 ej2* mutants (Soyk et al., 2017). However, in the VM, where *J2* and *EJ2* are not expressed, FUL2 and MBP20 probably interact with the protein products of the abundantly expressed *TM3*, *STM3* and *J* genes (Figure 3C). To confirm that these complexes can be formed and bind to CArG-boxes in the DNA, we performed EMSA experiments with FUL2 or MBP20 and the putative interaction partners. Because MADS-domain proteins can only bind to the DNA probe as dimers or tetramers (De Folter et al. 2005; Immink et al. 2009), a shifted probe in the assay indicates that a dimer has been formed. Since FUL2 and MBP20 do not form homodimers (Figures 1B and 3D, Supplemental Figures S1A and S7E), we could in most cases confirm

the formation of heterodimeric/tetrameric complexes by the gain of a probe shift. This was only problematic for the interactors that formed strong homodimers themselves (TM3 and J2), but for TM3, the addition of FUL2 or MBP20 resulted in a clear shift towards a tetrameric complex, confirming the Y2H data as well (Figure 3D and Supplemental Figure S7E). Thus, based on expression patterns of the genes and interaction capacity, FUL2 and MBP20 probably interact with TM3/STM3 and J in the VM to regulate flowering time, while it is plausible that FUL1, FUL2 and MBP20 form a complex with J2 and EJ2 to promote FM maturation. In addition, FUL1 and FUL2 may interact with the less abundant TM29 for this purpose.

Identification of Differentially Expressed Genes

Comparison of WT and quadruple mutant transcriptomes revealed 130 differentially expressed genes (DEGs) for the VM stage (103 up- and 27 down-regulated in the mutant), 125 for the TM stage (103 up- and 22 down-regulated), and 216 DEGs for the FIM stage (182 up- and 34 down-regulated), using FDR-corrected p-value <0.05 as a threshold for significance and a Log_2 fold change >1.0. These genes significantly overlapped between stages, with 23 genes differentially expressed in all three stages (Supplemental Figure S7B). Many more genes were upregulated in the mutant than there were down-regulated, pointing towards a general repressive function of SlFUL-containing complexes, in agreement with data from Arabidopsis *FUL* studies (Bemer et al. 2017; Balanzà et al. 2018; Ferrándiz et al. 2000). A large proportion of the DEGs are involved in metabolic processes, such as terpene synthesis or the phenylpropanoid pathway, but the corresponding genes were in general weakly expressed in the meristem (Supplemental Data set S1). Notably, the phenylpropanoid pathway is also controlled by *FUL1/2* in tomato fruits (Bemer et al. 2012), indicating that the regulation of some identified DEGs is probably of greater importance in other tissues. The DEG lists also contained several interesting genes that are possibly involved in flowering, although previously described tomato key regulators, such as *AN*, *FA*, *SFT*, *SP* and *S* were not among the DEGs (Supplemental Data set S1). We searched the list of DEGs for genes that may explain the flowering phenotypes instead, and identified a few homologs of known Arabidopsis flowering genes, such as *VRN1* and *AHL15*, which are involved in the regulation of flowering time and axillary meristem outgrowth, respectively (Levy et al. 2002; Karami et al. 2020). Also, the MADS-domain factors *TM3* and *SlMBP13* were significantly upregulated in the quadruple mutant in all three meristem stages. Most interestingly, however, is the identification of four CK signalling genes as targets of the *SlFULs*.

The SlFUL proteins repress negative regulators of cytokinin signalling

Compelling evidence shows that CK is required for SAM activity and FM initiation, and that the interplay of transcription factor regulation and CK signalling controls SAM size and activity (Kurakawa et al. 2007; Bartrina et al. 2011; Han et al. 2014). Moreover, a recent report showed that the CK reporter TCSv2 is highly expressed in tomato reproductive meristems (Steiner et al. 2020). In our list of DEGs, we identified several genes involved in CK signalling, namely three *CKXs*, *CKX5/6/8* (naming according to Matsuo et al. (2012)), and one *type-A ARABIDOPSIS RESPONSE REGULATOR* (*ARR*), *ARR16*. CKXs irreversibly degrade active CKs and type-A ARRs function as negative regulators of the CK response (Brownlee et al. 1975; D'Agostino et al. 2000; McGaw and Horgan 1983). *CKX6* and *CKX8* were upregulated in the VM and TM stages of the mutant, but not in the FIM stage, while *CKX5* and *ARR16* were only upregulated in the TM stage (Figure 4A). We confirmed their differential expression with RT-qPCR in independent samples (Supplemental Figure S8A). Therefore, upregulation of *CKX5/6/8* and *ARR16* in the VM and TM stages will probably result in a reduced CK content and responsiveness. We further investigated whether FUL2 and MBP20 can directly repress *CKX*/*ARR* gene expression by binding to their promoters. We therefore scanned the up- and downstream regions of the *CKX*/*ARR* genes for CArG-box motifs, the binding sites for MADS-domain proteins (Kaufmann et al. 2009; Aerts et al. 2018). Putative CArG-boxes were present in all differentially expressed *CKX*/*ARR* genes (Supplemental Figure S8B). To test whether FUL2 and MBP20 can bind to these motifs, we performed EMSAs using fragments containing these CArG-boxes as native probes. Because MADS-domain proteins need to form a dimer to bind to the DNA, we tested TM3-FUL2 and TM3-MBP20 heterodimers, as these proteins form strong heterodimers in yeast and are probably interacting in the VM/TM. In addition, as shown above (Figure 3D), the FUL2/MBP20-TM3 tetrameric complex can be easily distinguished from the TM3 homodimeric complex in EMSA assays. We detected clear shifts for all tested regulatory fragments (Figure 4B), suggesting that the FUL2-TM3 and MBP20-TM3 heterodimers can physically bind to the tested *CKX*/*ARR* genes. To investigate whether the CArG-box is essential for the binding, we also generated mutated probes, in which the CArG-box was mildly perturbed by a single-nucleotide mutation in the centre of the motifs. This probe mutation abolished or reduced the binding in all cases except for *CKX5* (Figure 4B), confirming the importance of the CArG box for the binding of the heterodimers. To determine whether FUL2 and MBP20 both play a role in repressing the CK signalling genes, we harvested meristems from *ful2* and *mbp20* single mutants and performed RT-qPCRs to determine the upregulation of the *CKX/ARR* genes. Upregulation was

visible in both single mutants but was in the VM stage more distinct in the *ful2* mutant than in the *mbp20* mutant (Figure 4C), in line with the higher expression of *FUL2* at this stage (Figure 3B). In the TM stage, both mutants showed a similar mild upregulation. The upregulation in the single mutants was considerably weaker than in the quadruple mutant, reflecting the partially redundant functions of both genes. To test our hypothesis that the late primary shoot flowering in the quadruple mutant is due to delayed doming as a result of impaired CK accumulation, we investigated the CK activity in the meristem of WT Moneyberg plants using the TCSv2:GUS reporter (Steiner et al., 2016). At the apex of the primary shoot, the CK signal was low in the early- and late VM stage, but intense in the TM stage (Figure 4D), indicating that an accumulation of CK had occurred, probably inducing meristem doming. To test whether CKX activity could indeed influence flowering time, we created two independent CRISPR mutants for the highest expressed gene, *CKX6*, and compared the flowering time of the homozygous T1 mutants with that of the WT (Supplemental Figure S8C and Figure 4E). The *ckx6* mutant showed a mild, but significant acceleration of flowering time, consistent with a role of CKX genes in the regulation of TM development right after the initiation of transition by upstream flowering signals. These results suggest that both FUL2 and MBP20 directly bind to the promoters of the *CKX5/CKX6/CKX*8 and *ARR16* genes to repress their expression and thereby upregulate CK signalling in the VM at the start of the transition to flowering.

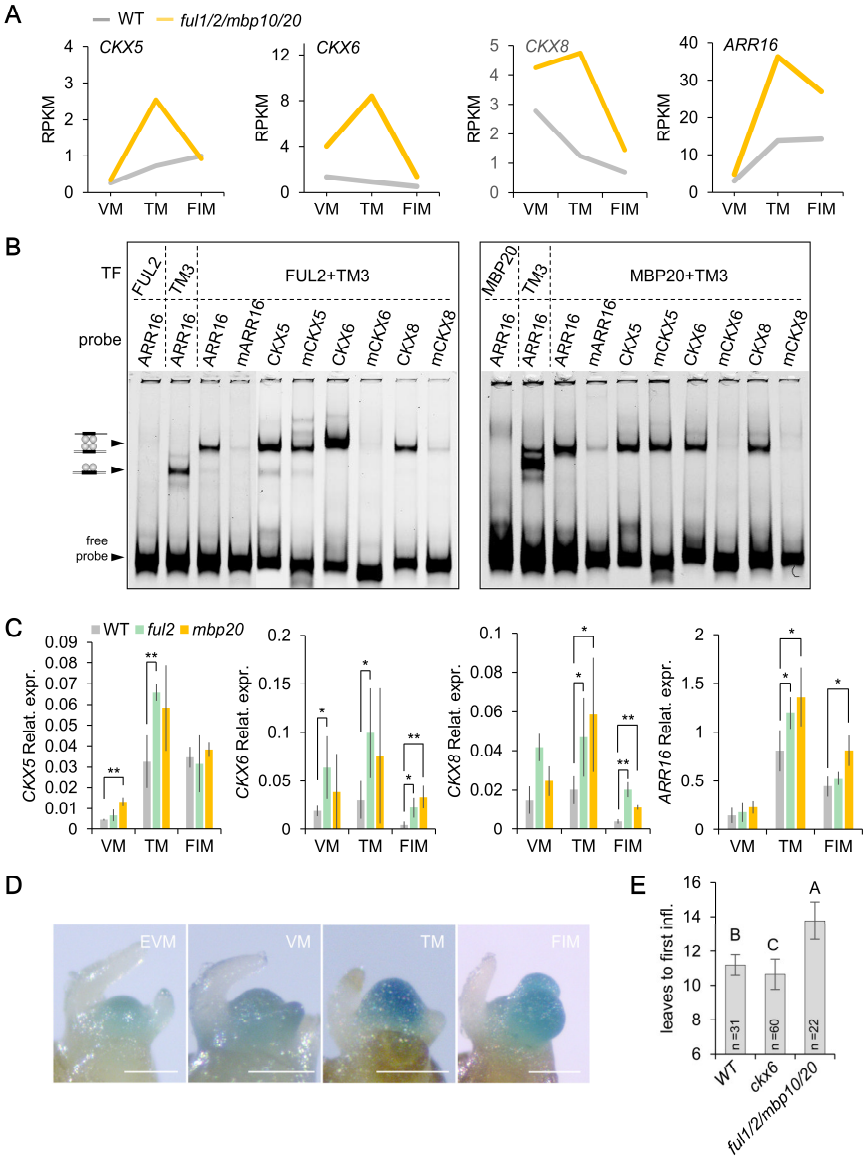

Figure 4. ***FUL2* and *MBP20* regulate *CKX5/6/8* and *ARR16* expression. (A)** Normalized gene expression (RPKM) for *CKX5/6/8* and *ARR16* across vegetative and reproductive meristem stages. **(B)** EMSA assays showing that FUL2-TM3 directly binds to promoter fragments of *CKX5/6/8* and *ARR16 in vitro*. The arrow indicates the shift of the probe caused by the binding of FUL2-TM3. The mutated versions of the promoter fragments are indicated with an 'm'. TF: transcription factor. **(C**) Expression of *CKX5/6/8* and *ARR16* during SAM transition in wild-type, *ful2* and *mbp20* obtained by qRT-PCR. **(D)** Staining of TCSv2:GUS in meristems during floral transition. **(E)** Quantification of primary shoot flowering for wild-type, *ckx6* and *slful* quadruple mutant plants. n: number of individual plants. In C, the values shown

(mean ± SD) are the average of three replicates. Significant differences were calculated using one-tailed Student's t test (*, ≤ 0.05 and **, P ≤ 0.01) in C, and one-way ANOVA followed by a post hoc LSD test in E, and different letters indicate the difference at P<0.01 level. VM: vegetative meristem; TM: transition meristem; FIM: floral meristem and inflorescence meristem. EVM: early VM.

Identification of downstream genes involved in the repression of branching

Searching for DEGs potentially involved in the flowering phenotype of the quadruple mutant, we found very few genes that could be associated with the inflorescence branching phenotype. However, as mentioned above, the primary shoot FIM, which was harvested for the RNA-seq, only rarely gave rise to branched inflorescences. Ubiquitous branching in the quadruple mutant was only observed in the inflorescences of the sympodial units, from the first sympodial unit onwards. Therefore, we performed an additional RNA-seq experiment to compare the transcriptomes of WT and quadruple mutant, harvesting the same mixed FM/IM from the sympodial shoot as sampled for the primary shoot, designated SFIM (Supplemental Figure S9A). This experiment, with the same set-up as described above, revealed 121 differentially expressed genes (DEGs), of which 96 were upregulated and 25 downregulated in the quadruple mutant. Previously reported key regulators of branching, such as *S*, *FA* and *AN*, were not in the list of DEGs. The expression of the *SP* gene, which suppresses the reproductive transition of the sympodial shoot meristem (Thouet et al. 2008; Pnueli et al. 1998), was remarkable, as it varied considerably between samples. (Supplemental Figure S9B). To identify genes possibly responsible for the inflorescence branching, we searched for flowering-related genes that were differentially regulated in the SFIM samples, but not in the FIM samples (Figure 5A). Four transcription factors were identified that may be involved in the regulation of FM maturation: *APETALA 2b* (*AP2b*) (Karlova et al. 2011), *AP2c*, *AGL6* and *TM29* (Figure 5B). Only for *AP2b*, the differential expression could be confirmed by RT-qPCR in independent samples (Supplemental Figure S9C). *AP2*-like genes are angiosperm-wide regulators of both meristem development and flowering, controlling for example stem-cell maintenance in the Arabidopsis SAM (Würschum et al. 2006), and floral and spikelet meristem initiation/termination in maize (*Zea mays L.*) (Chuck et al. 2008), in addition to their 'floral' roles in sepal/petal development and repression of the C-function (Morel et al. 2017). In addition to these specifically differentially expressed genes, two other genes that are also upregulated in the primary shoot FM, but to a lesser extent (Supplemental Data set S1), are probably candidates to explain the branching phenotype as well. Mutation of the first one, *TM3*, results in reduced inflorescence

branching in the *ej2 j2* mutant background, implying that higher expression of *TM3* will cause enhanced branching (Alonge et al. 2020). The other gene is a close homolog of Arabidopsis *AHL15*, which suppresses axillary meristem maturation (Karami et al. 2020). If tomato *AHL15* is also repressing meristem maturation, this could contribute to the enhanced branching phenotype. In conclusion, *FUL1*, *FUL2* and *MBP20* do not seem to regulate inflorescence branching by modifying the expression of the key regulators *S*, *FA* or *AN*, but we identified several other downstream transcription factors that may be involved.

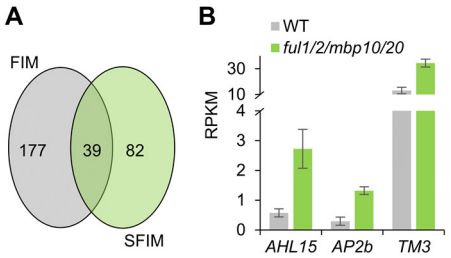

Figure 5. Gene expression in floral meristems of the sympodial shoot in WT and *slful* quadruple mutants. **(A)** Venn diagram showing the overlap of differentially expressed genes (DEGs) in the IM/FM (FIM) of primary shoot and sympodial shoot (SFIM). **(B)** Normalized gene expression (RPKM) of DEGs of interest in WT and quadruple mutant SFIM. The values shown (mean ± SD) are the average of three replicates.

FUL1 expression is regulated by FUL2 and MBP20

Despite the high expression of *FUL1* in the TM and FIM and the branching phenotype in the *ful1* single mutants, the *ful1* mutation does not enhance the branching phenotype of the *ful2 mbp20* mutant (Figure 2E, Supplemental Figure S4G). The considerable down-regulation of *FUL1* in the quadruple mutant may explain this apparent discrepancy (Figure 6A, Supplemental Figure S10A), and indicates that the gene is induced by FUL2, MBP20 and/or by itself via a positive (auto-)regulatory loop. Because the expression of *FUL1* is low in the VM, FUL2- and/or MBP20-containing complexes may need to bind to the CArG-boxes in the *FUL1* regulatory region to upregulate its expression in TM and FM/IM. To test this and determine the separate effects of FUL1, FUL2 and MBP20 on *FUL1* regulation, we performed RT-qPCRs in the corresponding single mutants (Figure 6B). Downregulation was observed in all three single mutants, particularly in the FIM stage, but the transcript reduction was most severe in the *ful1* mutants. The lower *FUL1* mRNA level in the *ful1* mutant may be caused by nonsense-mediated mRNA decay (NMD) as a result of the premature stop codon. However, it could also be the result of abolished *FUL1* autoregulation, or a combination of both decay and disturbed autoregulation. At this point, we cannot

discriminate between these possibilities. It is clear, however, that both FUL2 and MBP20 positively regulate *FUL1* expression. There are four CArG-boxes in the upstream region of *FUL1* that can probably be bound by MADS-domain complexes (Supplemental Figure S10B). To test whether FUL2 and MBP20 can bind, we performed EMSAs with TM3-FUL2 and TM3-MBP20 dimers and observed clear binding to the CArG-box containing probes (Figure 6C), suggesting that *FUL1* depends on FUL2 and/or MBP20 for maximal expression in the TM and FM stages.

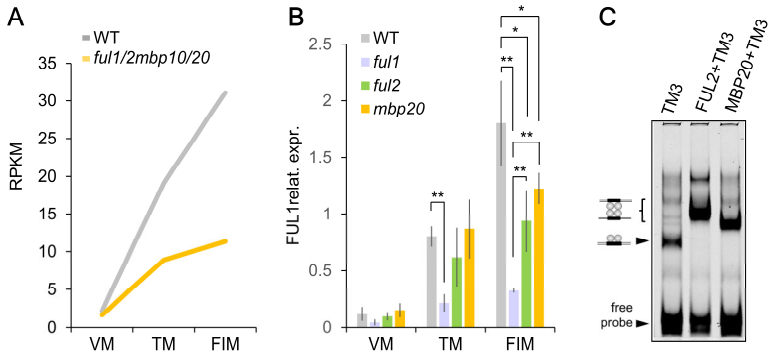

Figure 6. FUL2 and MBP20 regulate *FUL1* expression. (A) Normalized gene expression (RPKM) of *FUL1* across vegetative and reproductive meristem stages in wild-type (WT) and quadruple mutant. **(B)** FUL1 expression during SAM transition in WT, *ful1*, *ful2* and *mbp20* tested by qRT-PCR**. (C)** EMSA assays showing that FUL2/TM3 and MBP20/TM3 directly bind to the promoters of *FUL1 in vitro*. Average and standard deviation of two independent replicates per stage. The arrow indicates the shift of the probe caused by the binding of FUL2-TM3 or MBP20/TM3. The values shown (mean ± SD) are the average of three replicates. Significant differences were calculated using one-tailed Student's t test (*, ≤ 0.05 and **, P ≤ 0.01). VM: vegetative meristem; TM: transition meristem; FIM: floral meristem and inflorescence meristem.

DISCUSSION

Subfunctionalization of the *SlFUL* genes

Following segmental or whole-genome duplication events, genes with new molecular functions can arise through sub- or neofunctionalization, resulting in divergence of biological functions. We show here that functional divergence also occurred for the *SlFULs* after their multiplication early in the Solanaceae lineage. In addition to their previously described roles in fruit development and ripening (Bemer et al. 2012; Wang et al. 2019), we unveil that *FUL1* and *FUL2* both regulate FM/IM development, albeit at different levels. *MBP20* regulates flowering together with *FUL2* but does not contribute to fruit development. We did not observe any phenotype for the *mbp10*

mutant, nor did the mutation enhance the phenotype in higher-order mutants. This suggests, together with its weak overall expression pattern and low number of protein-protein interactions, that *MBP10* may become a pseudogene. In line with this, *MBP10* lacks regulatory sequences in its first intron (Maheepala et al., 2019), and has a three amino-acid mutation in the I-domain, a region important for protein-protein interactions (Van Dijk et al. 2010). The loss of *MBP10* in other Solanaceae genera such as *Petunia* also hints in this direction (Maheepala et al. 2019).

Although previous overexpression studies suggested that *MBP20* functions in leaf development and *FUL2* in stem development and secondary growth (Burko et al. 2013; Wang et al. 2014a; Shalit-Kaneh et al. 2019), we did not observe aberrant phenotypes in these tissues in our knockout mutants. The most probable explanation for this discrepancy is the use of the Cauliflower 35S promoter in the previous experiments (Wang et al. 2014a; Shalit-Kaneh et al. 2019), resulting in ectopic expression and mis-regulation of target genes at a position where *FUL2* and *MBP20* are usually not expressed. Overexpressing MADS-domain proteins or dominant-negative forms of MADS proteins can also perturb/block complexes of interaction partners in other tissues, leading to more severe phenotypes. However, another possibility is that *FUL2* and/or *MBP20* function redundantly with other MADS proteins in the investigated tissues. *AtFUL* and *SOC1* act redundantly in the regulation of secondary growth (Melzer et al. 2008), and *FUL2* may thus function redundantly with (*S*)*TM3* in the tomato stem as well. In conclusion, the four *SlFULs* underwent a functional divergence during evolution, but together retained functions in both inflorescence and fruit development. It is possible that some functions have remained unidentified due to redundancy with other MADS-box genes.

The position of *FUL1* in the flower regulatory network

FUL1 appears to act differently from *FUL2* and *MBP20* in the meristems. It is only weakly expressed in the VM, and its high expression in TM and FM probably depends on *FUL2* and *MBP20*, which are already expressed earlier in the VM and can bind to the *FUL1* promoter. (Auto-)regulatory loops are a common phenomenon in MADS-box gene regulation. For example, Arabidopsis *AP1* contains a CArG-box in its promoter, which can be bound by its own protein as well as by its paralog (*CAL*) to achieve high expression levels throughout different stages (Ye et al. 2016). Because of the delayed induction, *FUL1* does not regulate flowering time, but does contribute to the repression of inflorescence branching.

Interestingly, we found that *FUL1* has a premature stop codon at the C-terminus in the cultivar Moneyberg and many other cultivars. Although this truncation does not

alter *in vivo* dimer formation with other MADS-domain proteins (Supplemental Figure S2B), nor disturbs tetramer formation and DNA binding (Figure 1D), the C-terminus may be important for protein activity. It contains the highly conserved, but uncharacterized, FUL-specific PQWML motif (Litt and Irish 2003). Arabidopsis *ful* mutants complemented with a FUL copy with a mutation in this motif, were less able to rescue the silique phenotype than those transformed with a WT copy, suggesting that the motif is important for protein activity (McCarthy et al. 2015). Interestingly, the truncated allele has not been observed in wild relatives of tomato, and so probably first occurred after domestication (Supplemental Figure S2A). We did not find a correlation, however, between the presence of the *FUL1-ΔC* allele and any trait characterized by Roohanitaziani et al. (2020), but we cannot exclude that the allele has been selected during breeding, for example by conferring slightly larger inflorescences without severe branching.

The role of *FUL2* and *MBP20* in the tomato flowering network

Several previously identified tomato flowering genes were revealed to be functional homologs of Arabidopsis flowering genes, such as *SFT* (*FT*) and *FA* (*LFY*) (Molinero-Rosales et al. 1999; Molinero-Rosales et al. 2004; Lifschitz et al. 2006), indicating that at least part of the Arabidopsis flowering network is conserved in tomato. However, the knowledge of the regulatory network underlying the tomato sympodial flowering pathway is still fragmented and it is yet unclear whether homologs of many important players in Arabidopsis, such as *SOC1* and *FLC*, are important for tomato flowering as well. Here, we show that tomato *FUL*-like genes regulate flowering and inflorescence development in tomato, thereby adding a piece to the tomato flowering network puzzle. In Arabidopsis, *FUL* is a target of FLOWERING LOCUS D (FD)/FT and SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) proteins in the photoperiod pathway and the age pathway (Kardailsky et al. 1999; Wang et al. 2009; Jung et al. 2016), and functions partially redundantly with *AP1* in the promotion of flowering (Ferrándiz et al. 2000). We demonstrate here that *FUL2* and *MBP20* additively promote flowering similar to their homolog in Arabidopsis, but it is yet unclear whether they act downstream of SFT and the tomato SPLs as well. Within our set of DEGs, we did not identify any of the previously identified flowering regulators (e.g. FA, S, SFT, SP), further indicating that the *SlFULs* may act downstream of, or parallel to, these factors.

MADS-domain transcription factors bind to the DNA as dimers (De Folter et al. 2005), and since the SlFUL proteins cannot form homodimers, they need to heterodimerize with other MADS-domain proteins to regulate target gene expression. For the

regulation of flowering time, FUL2 and MBP20 probably form a complex with TM3, STM3 and J, because the corresponding genes are highly expressed in the VM/TM, and both *tm3 stm3* and *j* mutants display a small delay in flowering time (Szymkowiak and Irish 2006; Thouet et al. 2012; Alonge et al. 2020) similar to *ful2* and *mbp20*. Downstream of the *SlFULs*, we discovered several repressors of the CK pathway that are upregulated in the VM and TM stages of the quadruple mutant, probably resulting in reduced CK levels and signalling. In many species, the switch from vegetative growth to reproductive development is accompanied by cell division in the meristem, which results in meristem doming of the TM. We showed that this doming in tomato is accompanied by a high CK signal in the meristem, in agreement with the data of Steiner et al. (2020). This suggests that CK can positively regulate cell division during SAM doming to allow transition of the meristem. In line with this hypothesis, the reduced CK levels may inhibit SAM doming and thereby delay flowering. In Arabidopsis, CK deficiency through overexpression of *CKXs* diminishes SAM activity and indeed retards flowering (Werner et al. 2003). In addition, initiation of both the axillary meristem and the FM were shown to require a CK signaling (Wang et al. 2014b; Han et al. 2014). We show here that the tomato *CKX6* gene is also repressing flowering, and that its mutant displays early flowering, probably due to faster accumulation of CK in the meristem. The observed acceleration of flowering was mild, which can be explained by the role of the *CKX* genes downstream of key flowering regulators. Other CK repressors, such as *CKX8*, which is also expressed in VM and TM (Figure 4A), may have a partial redundant function and may increase the mutant phenotype when mutated in the *ckx6* mutant background. We did not further investigate the role of the *ARR16* gene, but its upregulation in the quadruple mutant may also add to impaired CK signalling and delayed TM development. In conclusion, our data provide evidence that *FUL2* and *MBP20* promote flowering through indirect regulation of CK levels by directly repressing *CKX* genes in the VM and TM (Figure 7).

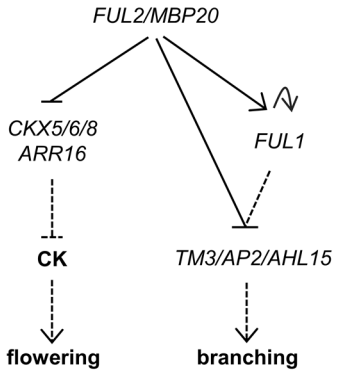

Figure 7. Model of SlFUL regulation of flowering time and inflorescence branching in tomato. The connections between the different regulators are based on the results of this study and other work described in the text. Solid lines display confirmed interactions, while dashed lines represent putative interactions. CK is cytokinin.

In other species, such as Arabidopsis and Petunia (*Petunia hybrida*), *AP1/FUL*-like genes are involved in the establishment of FM/IM identity, and combinatorial mutations result in a loss of identity, leading to a non-flowering phenotype. The Arabidopsis *ap1 cal* double mutant forms only IMs, because FM identity is lost, and additional mutation of *ful* aggravates this phenotype, leading to more vegetative structures (Ferrándiz et al. 2000). In Petunia, the four *FUL/AP1* genes appear to function redundantly in the establishment of FM/IM identity, and higher order mutants remain for a long time in the vegetative stage (Morel et al. 2019). In tomato, vegetative reversion occurs after a few flowers have formed in mutants of *MC* and *J*, suggesting that they are required for IM fate as well (Szymkowiak and Irish 2006; Thouet et al. 2012). We observed this phenotype only occasionally in the *slful* mutants, suggesting that the promotion of IM fate is mainly regulated by *J* and *MC*. It is possible, however, that the *SlFULs* act redundantly with *MC*, which would reflect the situation in Petunia.

The role of the *SlFULs* in the regulation of inflorescence architecture

We show that loss of function of *FUL1*, *FUL2* and/or *MBP20* results in a branched inflorescence that produces an increased number of flowers. The phenotype is variable, however, with some mutant inflorescences staying single-parous, while others form up to five branches. Our transcriptome analysis revealed that this branching is not caused by regulation of *S*, *AN* or *FA*, indicating that they function upstream of, or in parallel with, the SlFUL-containing complexes. Remarkable was the varying expression of *SP*, which may be involved in branching by the maintenance of IM identity (Supplemental Figure S9B). A probable explanation for this variation is variable SYM outgrowth in the leaf axils of the sampled meristems, although approximately 30 meristems were pooled for each sample. Because the branching phenotype is also highly variable, we cannot exclude that *SP* is somewhat regulated by the *SlFULs* in FM/IM, thereby exerting an effect on the branching phenotype. This would be similar to the repression of the legume *TFL1*-homolog by the euFULII clade proteins VEG1 (pea) or MtFUL1-c (Medicago) (Benlloch et al. 2015; Cheng et al. 2018; Zhang et al. 2021; Berbel et al. 2012). The branching phenotype of the quadruple mutant resembles that of the *j2 ej2* mutant (Soyk et al. 2017) and our microscopic analysis suggests that it is caused by delayed maturation of the FM as well. Given our *in vitro* interaction data, which show that the SlFULs can interact with J2 and EJ2, it is plausible that they act together in a complex to promote FM maturation and suppress inflorescence branching. Both *FUL1/FUL2/MBP20* and *J2/EJ2* are clearly expressed in the FIM in our data (Figure 3C), although our sampling method did not allow a clear distinction between FM and

flanking IM. However, the data from Park et al., (2012) are based on FMs that were completely isolated, and they describe high expression for *FUL1* and *FUL2* in the FM, while Soyk et al. (2017) describe the same for *J2* and *EJ2*, in agreement with an important role of a FUL1/FUL2/MBP20-J2/EJ2 complex in the regulation of FM maturation. Genetic experiments revealed that the *j2 ej2* mutant phenotype is additive to that of *s*, indicating that *J2* and *EJ2* function separately from the *S* gene (Soyk et al., 2017), and the same probably accounts for the *SlFULs*.

To identify genes downstream of the *SlFULs* that could explain the branching phenotype in the quadruple mutant, we performed transcriptome analysis in the FM/IM of the first sympodial unit. Our transcriptome analysis of the SFIMs unveiled several genes encoding transcription factors that were not differentially regulated in the FIM, or to a much lower extent. Since we observed the branching phenotype a few times in primary inflorescences of higher order mutants, the DEGs that were more prominent in the SFIM compared to the FIM, *TM3* and *AHL15*, could explain the higher frequency of branching in the sympodial shoot inflorescences by a dosage effect. *TM3* is an interesting candidate, because its expression is high in VM and TM, but drops in FIM. This suggests that TM3 is repressing FM maturation, in line with the observation that the *tm3 stm3* double mutation represses the enhanced branching phenotype of *j2 ej2* (Alonge et al. 2020). The upregulation of *TM3* will thus delay FM maturation and thereby enhance branching. Indications for the involvement of the other genes, *AHL15 and AP2b*, rather comes from research in Arabidopsis and maize. In Arabidopsis, *AHL15* represses meristem maturation in the axillary buds (Karami et al. 2020), while *AP2*-like genes regulate meristem development in both Arabidopsis and maize (Würschum et al. 2006; Chuck et al. 2008). Which of these downstream factors is most important for the increased-branching phenotype still needs to be determined with future genetic experiments and localization studies to establish what their function is in either the FM or the IM.

MATERIALS AND METHODS

Plant materials and growing conditions

Tomato cv. Moneyberg was used for the *Agrobacterium tumefaciens*-mediated transformation experiments (Van Roekel et al. 1993). Tissue culture was conducted in a growth chamber with 16 h light and 8 h dark at 25 °C. Plates were placed on shelves with either Philips TL 830 light tubes or Luxalight LED strips Neutral White 4300K, both with a light intensity of 60µE at the plate level. After rooting, the plants were transformed to rockwool blocks, watered with 1g/L Hyponex solution and cultivated in a 21 °C growth chamber (16h light/8h dark) under similar light conditions,

(light intensity of 70μE). 25-day old plants were moved to the greenhouse and grown under ambient temperatures and natural light, supplemented with artificial sodium lights.

RT-qPCR analysis

For RT-qPCR analysis of *SlFULs* expression, root, shoot, leaves, flower organs, and fruits of different stages were harvested from WT tomato plants. RNA was extracted with a CTAB/LiCl method (Porebski et al. 1997), DNase treated with Ambion Turbo DNase (AM1907) and cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed with the iQ SYBR Green Supermix from Bio-Rad with a standard 2-step program of 40 cycles, annealing at 60 °C. Primer efficiencies were tested beforehand and only primer pairs with equal efficiencies were compared. *CAC* was used as a reference gene (all primer sequences are listed in Supplemental Table S1).

Yeast two-hybrid (Y2H)

Protein–protein interaction assays in yeast were performed using the GAL4 System using Gateway vectors as described (De Folter and Immink 2011). The coding sequences for bait proteins and prey proteins were cloned into the pDEST32 and pDEST22 vectors respectively, and the vectors were transformed into the PJ69-4A and PJ69-4α yeast strains. The interaction screen was performed using -LWH dropout medium, supplemented with 3 mM 3-amino-1,2,4-triazole (3-AT) or -LWA dropout medium. Plates were incubated for 5 days at RT. All primer sequences used for cloning are listed in Supplemental Table S1.

CRISPR construct generation and stable tomato transformation

The *ful2* and *ful1 ful2* transgenic CRISPR lines have been previously generated (Wang et al. 2019). The constructs for all other lines were generated using GoldenGate cloning and the MoClo toolkit according to (Weber et al. 2011). Briefly, each gRNA was fused to the synthetic U6 promoter as U6p:gRNA, and cut-ligated in a Level 1 vector. Level 1 constructs pICH47732-NOSpro:NPTII:OCST, pICH47742-35S:Cas9:NOST, pICH47751-35S:GFP:ter35S, pICH47761-gRNA1, pICH47772-gRNA2, pICH47781-gRNA3 and the linker pICH41822 were cut/ligated into the level 2 vector pICSL4723 as described. After confirming the constructs, the plasmids were transformed into Agrobacterium strain C58C1. All primers are listed in Supplemental Table S1. The above constructs were introduced into tomato cv

Moneyberg by *Agrobacterium tumefaciens*-mediated transformation (Van Roekel et al. 1993). Homozygous T1 or T2 transgenic plants were used for phenotypic and molecular characterization.

Meristem imaging

Shoot apices were dissected from young plants using a forceps and older leaf primordia were removed to expose meristems under the stereomicroscope. Immediately after dissection, live meristems were imaged using an euromex scientific camera. To measure meristem size, dissected meristems were imaged under the stereomicroscope (Stemi 508, Zeiss) with a coupled camera (AxioCam IC, Zeiss Germany). Live meristems were imaged immediately after dissection. The SAM size was measured as the maximum width between leaf primordia using Leica Application Suite v4.9 software. CryoSEM images were prepared and imaged at the Wageningen Electron Microscopy Centre on the Magellan 400.

Fruit phenotyping

The second and third inflorescences were used for fruit phenotyping. For each inflorescence, only six flowers were kept and vibrated at anthesis to guarantee successful pollination. Individual fruits were harvested at breaker +7 (±1 day) for diameter and Brix measurements. Locule number was counted upon fruit cutting for Brix measurements. The Brix measurements were performed in duplo per fruit with an Atago PR-32α digital refractometer.

Meristem transcriptome profiling

The domesticated tomato (S. lycopersicum) cultivar Moneyberg and the homozygous *ful1 ful2 mbp10 mbp20* mutant generated in the Moneyberg background were used for transcriptome profiling. For each biological replicate sample, a batch of plants was grown and from each plant, the primary shoot meristem was harvested, either in the VM, TM or FM stage. The VM sample contained late VM meristems, just before transition (WT 9-leaf stage; quadruple mutant 12-leaf stage). For the SFIM samples, the first FIM from the sympodial shoot was harvested. About 60 plants were grown per batch (to harvest >30 meristems). All stages were harvested in triplicate for both the WT and quadruple mutant plants. The batches for the different replicates were grown in the greenhouse sequentially. Meristems were dissected using a stereoscope, and tissue was processed for RNA stabilization using an acetone fixation technique (Park et al. 2012). RNA was

extracted using the PicoPure RNA Extraction kit (Arcturus). More than 30 meristems were collected for each sample, yielding 1~3 µg RNA, which was enriched for mRNA and processed into cDNA libraries using the Illumina TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina). After quality control (Qubit and Fragment Analyzer), samples were sequenced using Illumina NovaSeq 2x150 nt Paired End sequencing. Samples were randomized across sequencing flow cells and lanes within flow cells. After quality control, all data were analyzed using the CLC work package. The raw data has been deposited in GEO under accession number GSE154419. For data validation, new batches of plants were grown and processed as described above, and the samples were analysed using RT-qPCR analysis (see Supplemental Table S1 for the primers).

Electrophoretic mobility shift assays (EMSAs)

FUL2 and MBP20 coding sequences were amplified from WT Moneyberg cDNA and cloned into pSPUTK (see Supplemental Table S1 for all primer sequences). The pSPUTK promoter allowed *in vitro* protein synthesis using the TnT® SP6 High-Yield Wheat Germ Protein Expression System (Promega) according to the manufacturer's instructions. The probe fragments consisted of a region of 80-100 bp with the canonical CArG-box in the middle and were amplified from genomic DNA. The mutated probe fragments were generated by overlapping PCR using primers that replaced one base pair in the middle of the CArG-box. EMSAs were performed essentially as described by Smaczniak *et al.* (2012) with minor modifications. Oligonucleotides were fluorescently labelled using DY-682. Labelling was performed by PCR using vector-specific DY-682-labelled primers followed by PCR purification with NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL). Gel shifts were visualized using a LiCor Odyssey imaging system at 700 nm.

GUS Histochemistry

The TCSv2:GUS reporter in the pART27 plasmid was obtained from the Weiss lab (Steiner et al. 2016) and transformed to tomato cultivar Moneyberg as described (Van Roekel et al. 1993). Histochemical analysis of the TCSv2:GUS reporter was performed according to (Soriano et al., 2014). Plant tissue was vacuum infiltrated for 5 min in a solution containing 2.0 mM potassium ferri- and ferrocyanide, and incubated overnight at 37 °C. Tissue was then cleared in 70% ethanol prior to imaging.

Accession numbers

FUL1, Solyc06g069430; FUL2, Solyc03g114830; MBP10, Solyc02g065730; MBP20, Solyc02g089210; J, Solyc11g010570; J2, Solyc12g038510; EJ2, Solyc03g114840; TM29, Solyc02g089200; MADS-RIN, Solyc05g012020; TM5, Solyc05g015750; TM3, Solyc01g093965; STM3, Solyc01g092950; SlMBP18, Solyc03g006830; TAG1, Solyc02g071730; SlMBP24, Solyc01g105800; SlMBP13, Solyc08g080100; SlMBP14, Solyc12g056460; SlMBP9, Solyc04g076680; SlMBP12, Solyc12g088090; SlMBP22, Solyc11g005120; MC, Solyc05g056620; AHL15, Solyc12g087950; AP2b, Solyc02g064960; AP2c, Solyc02g093150; AGL6, Solyc01g093960; CKX5, Solyc04g016430; CKX6, Solyc12g008900; CKX8, Solyc10g017990; ARR16, Solyc06g048930. Supplemental Data set S1 contains the accession numbers of the DEGs. The raw data of the RNA-seq experiments has been deposited in GEO under accession number GSE154419.

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AUTHOR CONTRIBUTIONS

M.B. conceived the project and designed the experiments; X.J. performed the qPCRs, tomato CRISPR/cas9 mutagenesis, RNA-seq experiments, EMSAs, SEM analysis, and CK reporter identification; K.R, G.L. and J.H.L. did the Y2H analyses; V.V. measured the fruit phenotypes; R.A.d.M. analysed *FUL1* alleles in cultivars and assisted with the RNA-seq analysis; G.C.A. and M.B. supervised the project; M.B. and X.J analysed the data, prepared the figures and wrote the article. All authors read and approved the final version.

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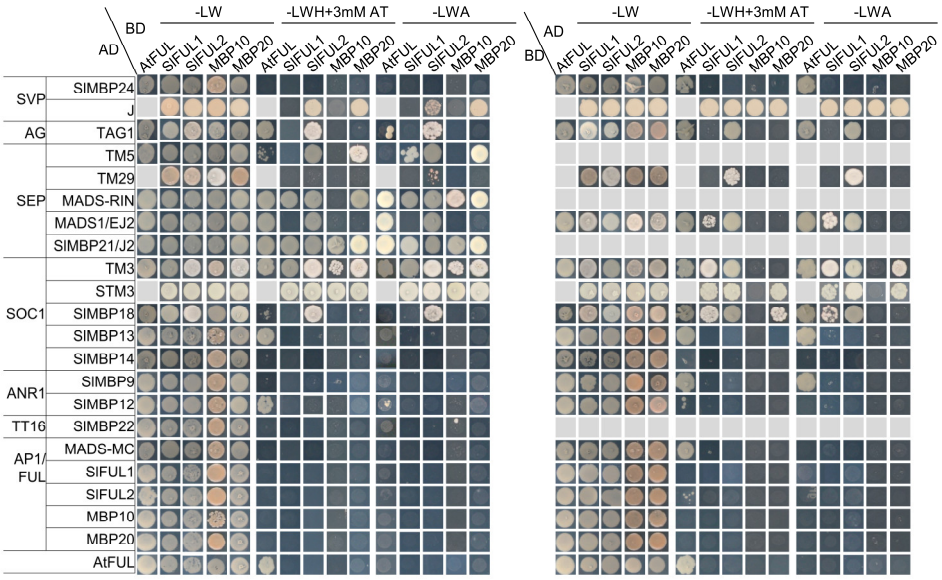
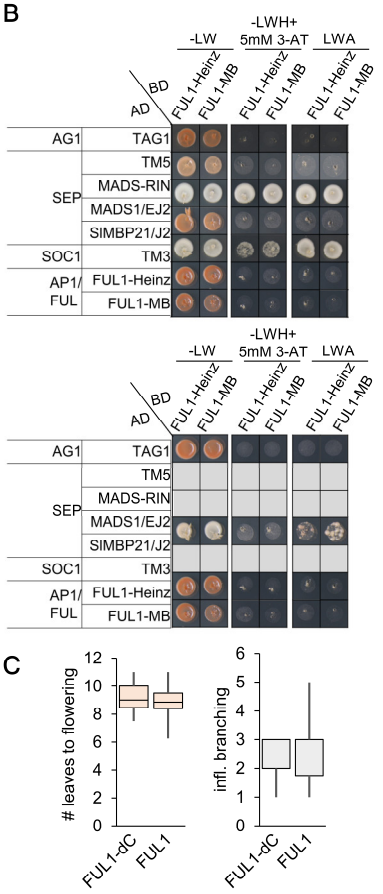


Supplemental Figure 1. **Yeast two-hybrid analysis of FRUITFULL-like proteins with MADS-box proteins from different subfamilies**. SVP: SHORT VEGETATIVE PHASE; AG: AGAMOUS; SEP: SEPALATTA; SOC1: SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1; ANR1: ARABIDOPSIS NITRATE REGULATED 1; TT16: TRANSPARENT TESTA16; AP1/FUL: APETALA1/FRUITFULL. L, leucine; W, tryptophan; H, histidine; A, adenine; 3-AT, 3-amino-1,2,4-triazole. Grey boxes indicate that the interaction was not tested, in most cases because the bait gave auto-activation.

A

Variety or Cultivar Name	Deletion present
Heinz 1706 (reference genome)	.
Moneymaker	X
Moneyberg	X
Ailsa Craig	X
Rutgers	X
Galina (Galina's yellow; Galina's cherry)	.
Ponderosa	.
John's Big Orange	.
All Round	X
Sonato	X
Cross Country	.
Lidi	.
Momatero	.
Large Pink	.
Jersey Devil	.
Polish Joe	.
Cal J TM VF	.
The Dutchman	.
Black Cherry	.
ANTO	X
Winter Tipe (nor)	X
Chang Li	.
Belmonte	X
Tiffen Mennonite	.
Wheatley's Frost Resistant	X
Chih-Mu-Tao-Se	X
ES 58 Heinz	.
Dolmalik	X
Large Red Cherry	X
Porter	X
Bloody Butcher	.
Brandywine	.
Dixy Golden Giant	.
Giant Belgium	X
Kentucky Beefsteak	.
Marmande VFA	X
Thessaloniki	X
Watermelon Beefsteak	.
S. pimpinellifolium	.
S. chmielewski	.
S. cheesmaniae	.

B



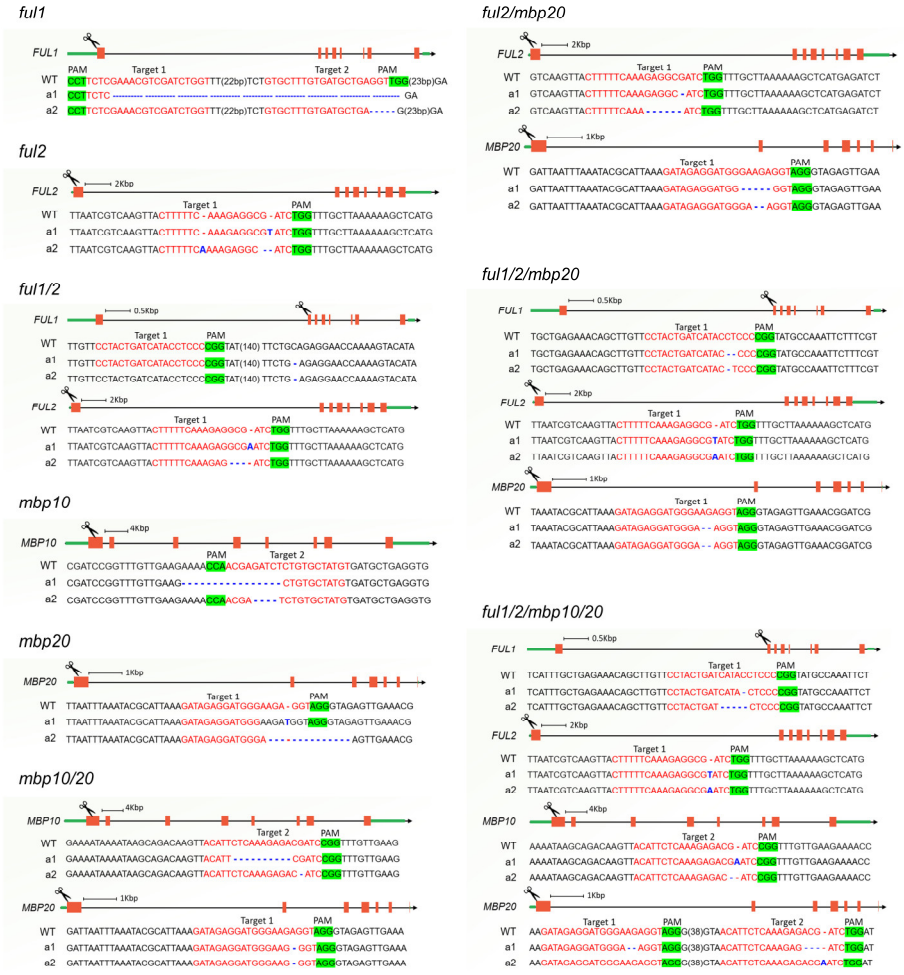
C

Supplemental Figure 2. The truncated FUL1 version is present in many cultivars but displays the same protein-protein interactions as the full-length reference protein. (A) Table showing the occurrence of the 1nt deletion allele in 37 resequenced cultivars and wild species. X = 1nt deletion is present. **(B)** Yeast two-hybrid screening using a subset of interaction pairs and both the full-length (Heinz) and truncated (Moneyberg) FUL1 protein. **(C)** Flowering and branching phenotype of the cultivars with truncated FUL1 (FUL1-dC) and FUL1. **(D)** (see next page) Table of features grouped for cultivars with FUL1-dC and cultivars with 'normal' FUL1.

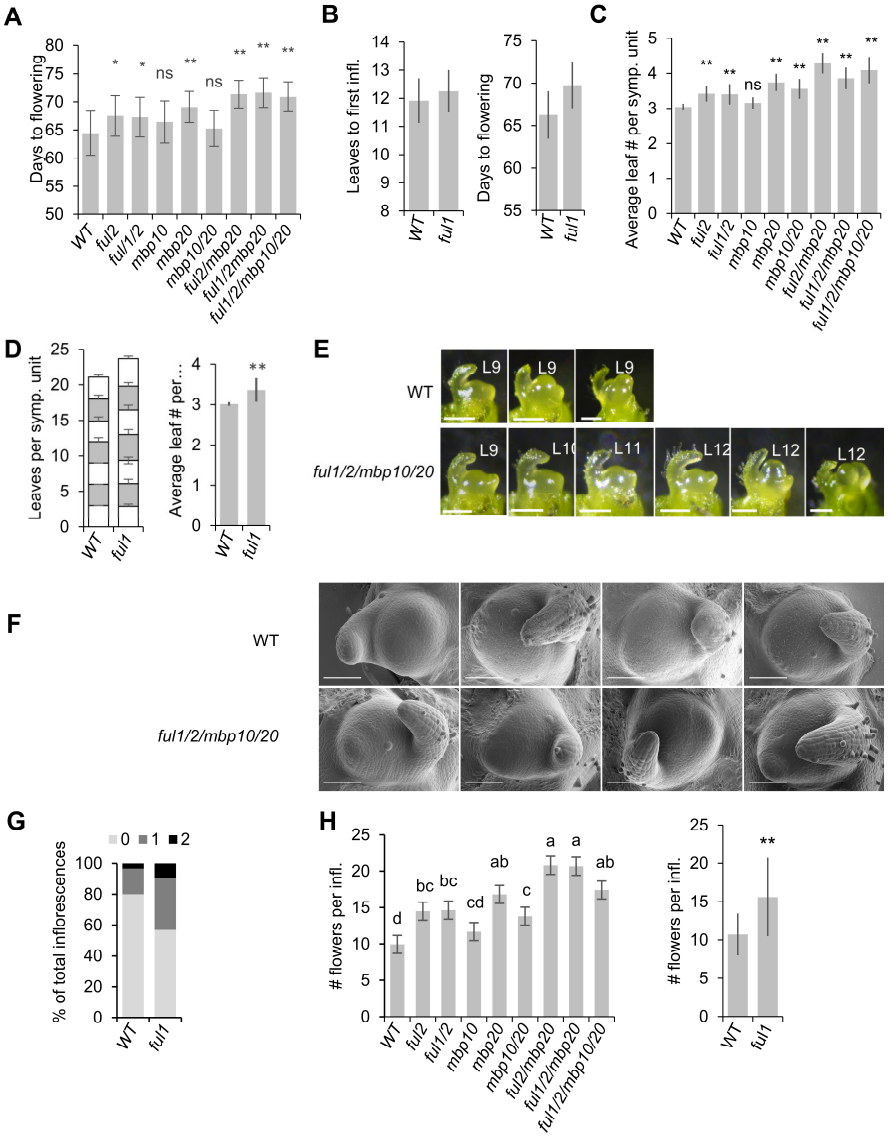
D

Genotype name	FUL allele	AZ category	Inflorescence branching	VOI	Flowering Time	Number of fruits	Fruit Weight (g)	Degrees Brix	Firmness (N)	Color	Shape
Moneymaker	dC	1.0	2.0	3.0	8.8	64.0	41.7	4.9	52.9	red	round
Alisa Craig	dC	1.0	2.0	1.0	8.8	66.5	49.6	4.1	55.4	red	round
Rutgers	dC	1.0	1.0	1.0	8.3	12.3	154.3	4.7	47.7	red	Ox-heart
All Round	dC	1.0	3.0	3.0	9.0	77.0	52.9	4.7	51.5	red	round
Sonato	dC	1.0	3.0	1.0	10.0	65.0	58.9	4.6	51.6	red	round
ANTO	dC	1.8	3.0	3.0	8.0	29.8	193.2	4.9	57.4	red	flat
Winter Tipe (nor)	dC	-	2.0	3.0	10.7	29.3	34.0	4.3	65.3	Green	round
Belmonte	dC	1.8	3.0	3.0	8.4	18.5	317.9	3.9	49.6	dark pink	flat
Wheatley's Frost R	dC	1.0	-	-	11.0	79.0	13.0	5.0	41.8	pink	rectangular
Chih-Mu-Tao-Se	dC	1.0	2.0	1.0	10.7	36.3	99.5	4.7	46.3	pink	flat
Dolmalik	dC	1.0	3.0	4.0	9.3	65.3	56.4	4.9	43.1	red	flat
Large Red Cherry	dC	1.0	2.0	4.0	9.0	106.5	25.3	5.2	49.3	red	round
Porter	dC	1.0	2.0	1.0	10.0	59.0	21.5	4.2	48.3	pink	round
Giant Belgium	dC	2.0	3.0	2.0	7.5	20.5	249.0	4.1	49.1	dark pink	flat
Mamande VFA	dC	1.3	3.0	1.0	8.5	35.3	89.8	4.4	51.0	red	flat
Thessaloniki	dC	1.0	3.0	1.0	9.3	18.8	127.3	4.7	39.7	red	flat
		1.2	2.5	2.1	9.2	48.9	99.0	4.6	50.0		
Galina	normal	1.0	1.0	1.0	9.2	190.5	13.3	6.0	53.5	orange	round
Ponderosa	normal	2.0	3.0	1.0	8.4	16.0	162.1	4.7	54.5	red/orange	round
Ponderosa	normal	-	3.0	2.0	8.9				59.6		
John's big orange	normal	1.0	3.0	1.0	6.3	32.0	56.6	4.0	52.8	orange	flat round
Cross Country	normal	1.0	1.0	1.0	9.2	75.5	36.6	4.2	37.4	red	round
Udi	normal	1.5	5.0	5.0	10.1	439.0	6.2	6.3	55.4	yellow	ovate
Momatero	normal	1.0	2.0	4.0	10.0	29.3	150.7	4.8	55.1	pink	flat
Large Pink	normal	1.0	3.0	1.0	7.7	19.8	244.5	3.7	49.0	pink	flat
Jersey Devil	normal	1.0	3.0	4.0	11.0	13.8	105.8	4.7	53.4	red	oxheart
Polish Joe	normal	2.0	3.0	3.0	8.2	27.8	265.9	3.9	59.8	pink	heart
Cal J TM VF	normal	2.5	1.0	1.0	8.0	52.5	46.3	4.7	47.9	red	round
The Dutchman	normal	1.5	3.0	3.0	9.8	18.5	306.7	4.6	51.9	pink	flat
Black Cherry	normal	1.0	3.0	1.0	8.9	187.5	14.7	6.4	48.5	dark pink	round
Chang Li "L esculer	normal	1.0	-	-	8.7	66.0	15.3	5.5	54.0	yellow	round
Tiffen mennonite	normal	1.0	3.0	3.0	8.5	23.3	142.9	4.7	56.7	pink	flat
ES 58 Heinz' L. escu	normal	1.0	1.0	1.0	9.5	18.5	99.4	3.5	41.8	red	round
Bloody Butcher	normal	1.3	3.0	4.0	9.5	50.8	43.8	5.4	52.8	red	flat
Brandywine	normal	1.0	1.0	1.0	9.5	14.3	231.4	4.7	43.3	dark pink	round
Dixy Golden Giant	normal	1.0	3.0	1.0	9.1	14.8	253.1	4.9	49.5	orange	flat
Kentucky Beefstea	normal	1.3	4.0	3.0	8.0	14.3	243.4	3.7	45.5	orange	flat
Watermelon Beefs	normal	2.0	3.0	4.0	8.4	5.8	305.7	5.1	51.0	pink	ovate
		1.3	2.6	2.3	8.9	64.5	137.2	4.8	51.1		
T-test		0.571999	0.732143	0.956789	0.420505	0.49979	0.375488	0.430103	0.578271		

Supplemental Figure 2 Continued.

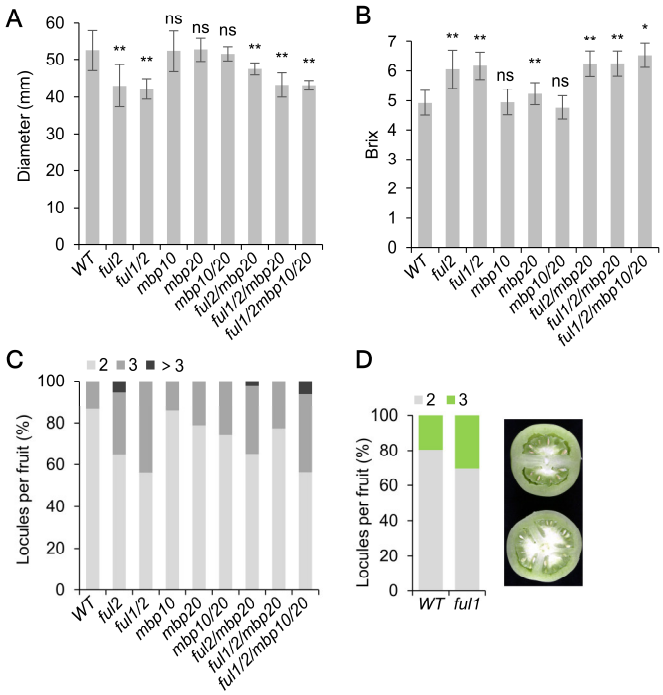


Supplemental Figure 3. Mutations of tomato *FUL*-like genes generated by CRISPR/Cas9. Two independent homozygous loss-of-function alleles (a1 and a2) with insertions or deletions in the target regions were selected for each mutant. The orange and green boxes indicate coding exons and UTR of the genes, respectively. Cartoon scissor indicates the target exons. The red font and green highlights indicate sgRNA targets and protospacer-adjacent motif (PAM) sequences, respectively. Numbers in parentheses show gap lengths. Blue dash and letter indicate deletion and insertion. The *ful2* single mutant and *ful1/ful2* double mutant lines were obtained from Wang et al., 2019.

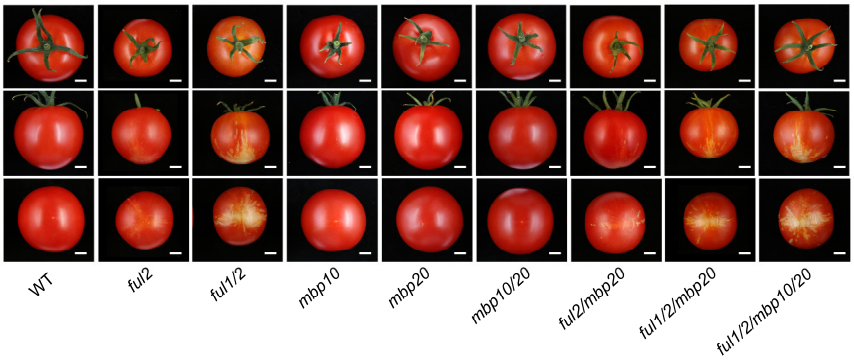


Supplemental Figure 4. *slful* mutants show delayed flowering in primary shoot and sympodial shoot. (A) Quantification of primary shoot flowering times indicated by days from seed sowing to first flower opening. **(B)** Flowering data of primary shoots for wild-type (WT) and the *ful-1* single mutant. **(C)** Average leaf number of the first five successive sympodial shoots. **(D)** Flowering data of sympodial shoots for WT and the *ful-1* single mutant. **(E)** A developmental series of shoot apical meristem of WT and quadruple mutant from the vegetative stage to floral transition. White bar: 200 µm. **(F)** Scanning electron micrographs of vegetative meristems of WT and quadruple mutants. white bar: 100 µm. **(G)** Inflorescence branching phenotype of WT and *ful1* mutant plants. **(H)** The quantification of the number of

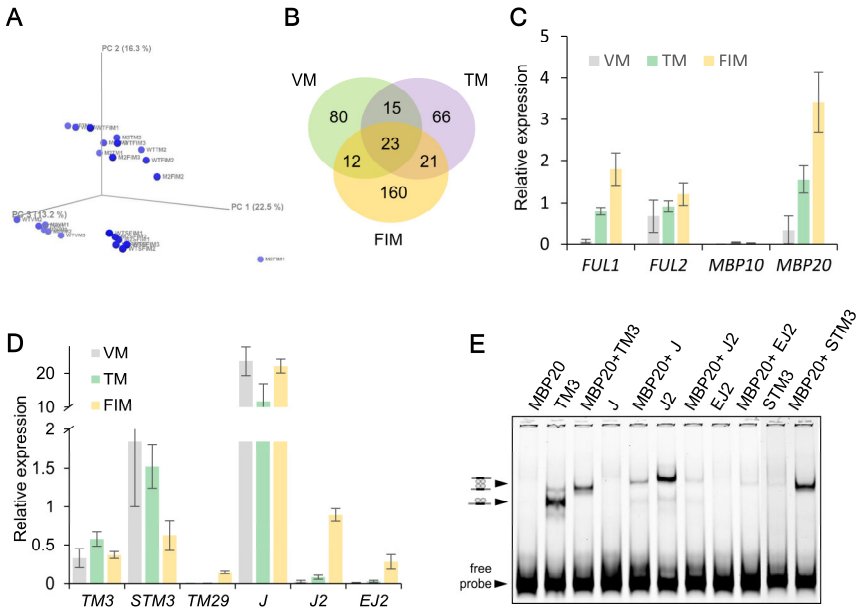
flowers per inflorescence. The data of B, D, F, and right panel of G were observed in independent experiment. Mean values (± SD) were compared to WT using one way ANOVA followed by a post hoc LSD test. Significant differences are represented by asterisks. (∗) P-value < 0.05; (∗∗) P-value < 0.01; ns, not significant. Six individual T2 offspring plants were analyzed per line and the data from the two different genotypes were combined for each mutant (e.g., 2x6 individuals for *ful2* etc.).



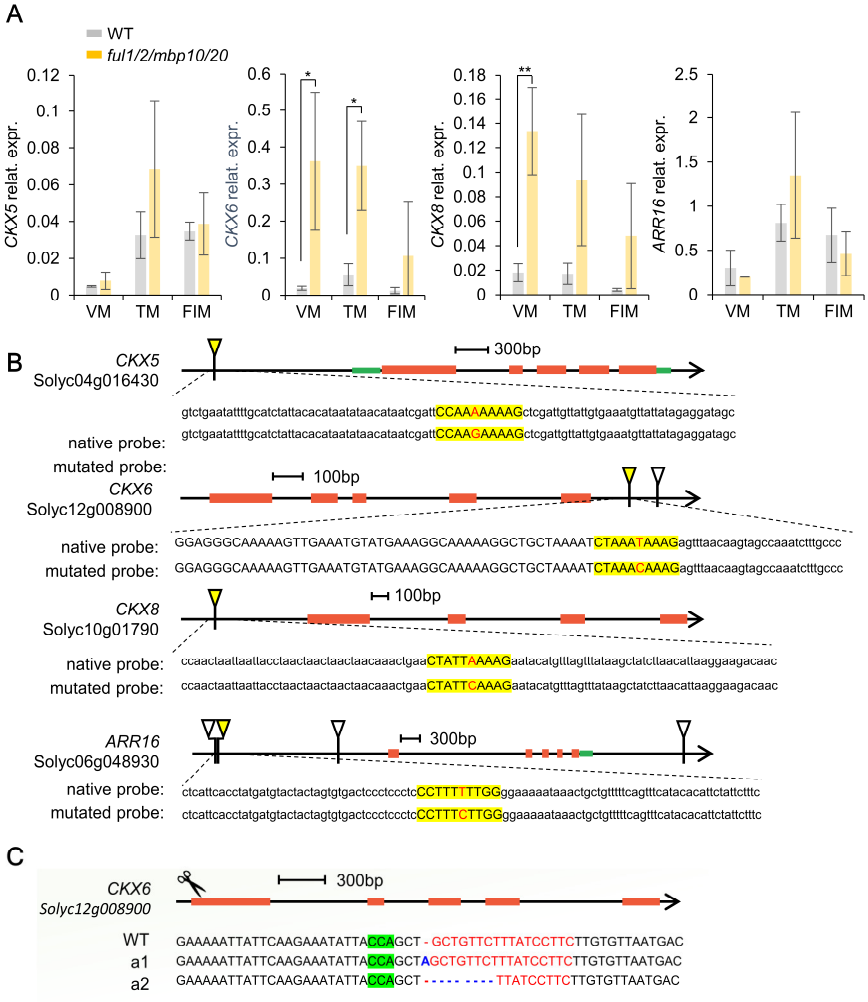
Supplemental Figure 5. Fruit phenotypes of the *slful* mutants. (A) Fruit diameter of wild-type (WT) and mutants. **(B**) Brix values of WT and mutants, indicating the soluble sugar contents in the fruits. Values of 16 to 40 fruits for each genotype were used. Mean values (± SD) were compared to WT using one way ANOVA. Significant differences are represented by asterisks. (∗) P-value < 0.05; (∗∗) P-value < 0.01; ns, not significant. **(C)** Proportion of fruits with a given locule number. 16-40 fruits per genotype were used.



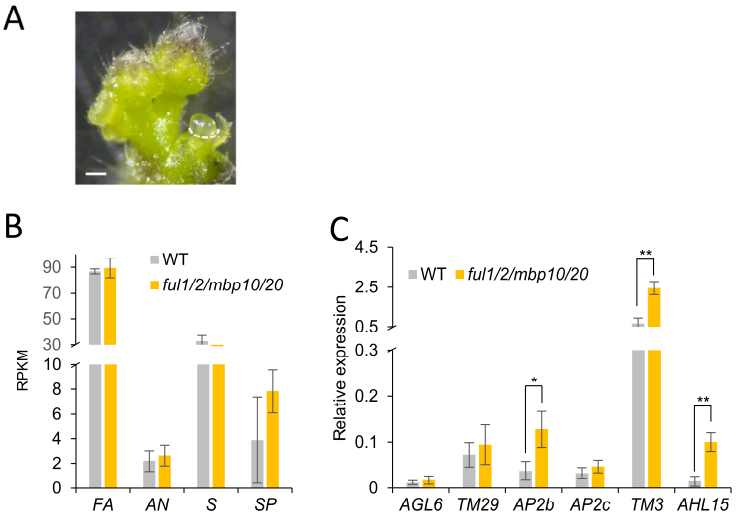
Supplemental Figure 6. Fruit phenotype of wild-type and *slful* mutants. Red ripe fruits were harvested for the picture. White bar: 1 cm.



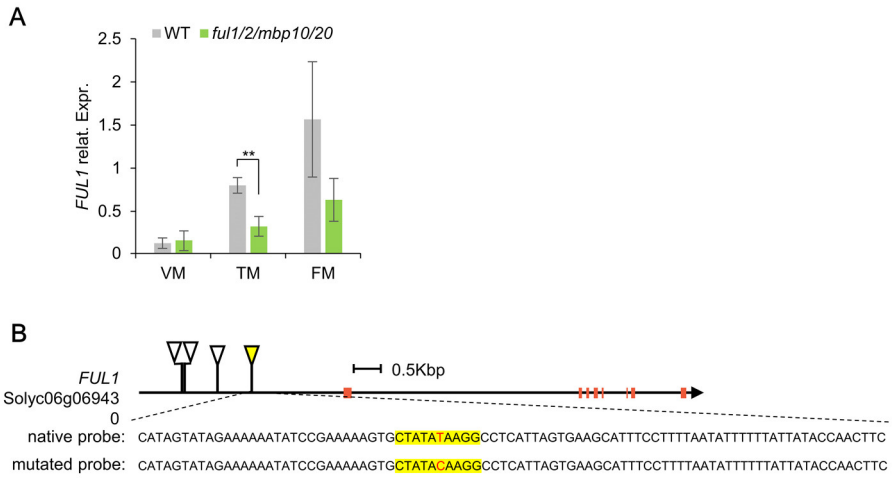
Supplemental Figure 7. Relative expression profiles of SlFUL and the MADS-box genes encoding their interacting proteins. **(A)** PCA of differentially expressed genes (DEGs) in the meristem of different stages of wild-type (WT) and *ful1/2/mbp10/20* (M2), determined by RNA-seq. **(B)** Venn diagram showing the overlap of DEGs in VM, TM and FM of WT and M2**. (C), (D)** Expression of *SlFUL* genes and MADS-box genes encoding *SlFUL* interactors in three successive stages of SAM transition obtained by qRT-PCR. The values shown (mean ± SD) are the average of three replicates. VM: vegetative meristem; TM: transition meristem; FIM: floral meristem and inflorescence meristem. SFIM: sympodial FIM.



Supplemental Figure 8. FUL2/MBP20 regulation of cytokinin negative regulators. (A) Expression *CKX5/6/8 and ARR16* in meristems of wild-type and quadruple mutant by qPCR. Significant differences were calculated using one-way ANOVA followed by a post hoc LSD test (*, ≤ 0.05 and **, P ≤ 0.01). VM: vegetative meristem; TM: transition meristem; FIM: floral meristem and inflorescence meristem. The values shown (mean ± SD) are the average of three replicates. **(B)** Schematic representation of *CKX5/6/8 and ARR16* genomic loci showing the FUL2 and MBP20 binding sequences of CArG motifs. The red and green boxes indicate exons and UTRs of genes, respectively. Triangles indicate CArG boxes and yellow-highlighted regions were tested by EMSAs. Nucleotides in red were changed in the mutated probes.



Supplemental Figure 9. Gene expression in the FM/IM of the first sympodial unit. (A) Manual microdissection of the FM/IM meristems of the first sympodial unit (SFIM) for transcriptome profiling. The white dashed line indicates the dissected tissue. White bar: 200 µm. **(B)** Normalized gene expression (RPKM) of *FA*, *AN*, *S*, *SP* in SFM of wild-type and quadruple mutant. **(C)** Expression of *AGL6*, *TM29*, *AP2b*, *AP2c*, *TM3* and *AHL15* in the SFM of wild-type and quadruple mutant obtained by qRT-PCR**.** The values shown (mean ± SD) are the average of three replicates. Significant differences were calculated using one-way ANOVA followed by a post hoc LSD test (*, ≤ 0.05 and **, P ≤ 0.01).



Supplemental Figure 10. FUL2/MBP20 regulation of FUL1. (A) *FUL1* expression in meristems of wild-type and quadruple mutant by qPCR. The values shown (mean ± SD) are the average of three replicates. Significant differences were calculated using one-way ANOVA followed by a post hoc LSD test (*, ≤ 0.05 and **, P ≤ 0.01). **(B)** Schematic representation of *FUL1* genomic loci showing the FUL2 and MBP20 binding sequences of CArG motifs. The red boxes indicate exons of genes. Triangles indicate CarG boxes and yellow-highlighted regions were tested by EMSAs. Nucleotides in red were changed in the mutated probe

Supplemental Table S1. Primer used in this study

Experiment	Gene	Primer sequence (5'-3')
Y2H	*FUL1*	**F:** AAAAAGCAGGCTTGGGAAGAGGAAGAGTCCA
		R: AGAAAGCTGGGTTTAATTATTAAGATGACGAAGCA
	FUL2	**F:** AAAAAGCAGGCTTGGGTAGAGGAAGAGTACA
		R: AGAAAGCTGGGTTTAACCGTTGAGATGTCGAAGCA
	MBP10	**F:** AAAAAGCAGGCTTGGGGCGGGGTAGGGTG
		R: AGAAAGCTGGGTTCATCCTTTGTTGTGGACATGGT
	MBP20	**F:** AAAAAGCAGGCTTGGGAAGAGGTAGGGTAGAGT
		R: AGAAAGCTGGGTTCATCCTTCGTTGCTGACGTGG
	SlMBP24	**F:** AAAAAGCAGGCTTGATGGTGAGACAAAAAATTCAG
		R: AGAAAGCTGGGTCTAAGGAAAAGCCAAGCATAACTTG
	J	**F:**AAAAAGCAGGCTTGGCTAGAGAAAAAATTCAGATCAAGAAAATA
		R:AGAAAGCTGGGTTTACTTTTTTTTTTCTCCTTCTTCTAATAACAT ACAAAAATAG
	TAG1	**F:** AAAAAGCAGGCTATATGGACTTCCAAAGTGATCTAACC
		R: AGAAAGCTGGGTTTAGACTAGTTGAATAGGGGGTTGG
	TM5	**F:** CACCATGGGAAGGGGTAGGGTTGAGCT
		R: TCAAGGCAACCAGCCAGCCA
	TM29	**F:** CACCATGGGTAGAGGAAGAGTTGAGCTGA
		R: TCACAGCATCCAACCAGGTATCA
	MADS-RIN	**F:** CACCATGGGTAGAGGGAAAGTAGAATTGAAG
		R: TCAAAGCATCCATCCAGGTACAA
	MADS1/EJ2	**F:** CACCATGGGAAGAGGAAGAGTTGAGCTTAAG
		R: TTAAAGCATCCATCCATGAATAAATC
	SlMBP21/J2	**F:** CACCATGGGAAGAGGAAGAGTAGAACTAAAGAG
		R: TTAGAGCATCCACCCTGGAATAAATC
	TM3	**F:** AAAAAGCAGGCTTGGTTCGAGGTAAAACCCAG
		R: AGAAAGCTGGGTTAGAGACGTCTTTCTCTGCAC
	STM3	**F:** AAAAAGCAGGCTTGGTTCGAGGTAAAACCCAGA
		R: AGAAAGCTGGGTAAGGGCTAAAAGTTAAACTCCATC
	SlMBP18/FYFL	**F:** AAAAAGCAGGCTTGGTGAGAGGAAAAGTAGAAATG
		R: AGAAAGCTGGGTCTATAAGCAGCGCATTTGAG
	SlMBP13	**F:** AAAAAGCAGGCTTGGTGAGAGGAAAAACTGAGT
		R: AGAAAGCTGGGTTTAAAGTGATGTAGGTAGTGTG
	SlMBP14	**F:** AAAAAGCAGGCTTGGTGAGGGGAAAAACTGAG
		R: AGAAAGCTGGGTTCATCTTTCAGGAAGTCCAA
	SlMBP9	**F:** AAAAAGCAGGCTTGGGGAGAGGTAAGATAGTG
		R: AGAAAGCTGGGTGGTTTTCACTGCGAATGC
	SlMBP12	**F:** AAAAAGCAGGCTTGGGGAGAGGAAAGATATTG
		R: AGAAAGCTGGGTTCCACTAATGCAGTGACAG
	SlMBP22	**F:** AAAAAGCAGGCTTGGGGAGAGGAAAGATAG
		R: AGAAAGCTGGGTTCAATCGTAGGTAGAGGGAG
	MC	**F:** AAAAAGCAGGCTTGGGAAGAGGAAAAGTTG
		R: AGAAAGCTGGGTTCATAGATGTTTATTCATGTT

Experiment	Gene	Primer sequence (5'-3')
RT-qPCR	*FUL1*	**F:** GTTTTGCCACAACAACTGGACTC
		F: GTTTTGCCACAACAACTGGACTC
		R: CTTGCTGCTGTGAAGAACTACC
	FUL2	**F:** CATGAGATCTCTGTGCTTTGCG
		R: ATCCTTTCCATGCAAGAGTCAGT
	MBP10	**F:** GAATTTCGGGGTTTAGAGAAACAGC
		R: GCTGGGAAATGGACTCGTGC
	MBP20	**F:** CACATTCTCACCACCAACTTCCTAA
		R: AGTGATGAGCCTGACCCGAT
	J	**F:** ATTGATCCTCCTCCTCAAGATGATG
		R: CTCTTCAGCCTGAGTAAGGTAGCC
	J2	**F:** GCTGGCAGACCTTCAAGAAAAG
		R: CCATTGTTTGTCCTCCATTATTTCC
	EJ2	**F:** AAGCAAATCAGGTCAAGGAAGACAC
		R: CCTCCATCTTCCCAACACAATCG
	TM3	**F:** GGAAGTTCTTGGGAGAAGGTCTG
		R: AGTCGCTCAACCTGTTCCTTG
	STM3	**F:** GCAATTGGAGCAGAGTGTCA
		R: TCGTCTCTCTTCATCTCCTCCA
	TM29	**F:** GTGGAATGAGGCTAACAAGGTTCT
		R: GACTTTGCTCACCACCACCC
	AGL6	**F:** TGTGAGGCTGAAGTTGCTCT
		R: GCAACAACGTTGGTACCTCTC
	AP2b	**F:** ACCCAAGCAACCTAGTCCAC
		R: CCGGAGAATGTAGGTGCGTT
	AP2c	**F:** TGGATATTGATTGGCAGCGC
		R: TGGAGAATGCAAGTGCGTCT
	AHL15	**F:** TTGAAGTCATCCGCCGTTCA
		R: TGGGTTTTTCCACGTGACCA
	CKX5	**F:** TGGGCAAGCATTCAAACATGG
		R: AGAACAGGTCAGCATTCTGC
	CKX6	**F:** CTAATGCTGGAATTAGTGGTCAAAC
		R: CATATCTTTGGAGCAAGTCATTAAT
	CKX8	**F:** CTTCCAAAGATTTTGGAAAGATC
		R: GATAGAAAGGCCATAAGAAAATTGA
	ARR16	**F:** AAGGGCGTTGGAGTACTTGG
		R: CCAGTCATTCCTGGCATGCA
	CAC	**F:** CCTCCGTTGTGATGTAACTGG
		R: ATTGGTGGAAAGTAACATCATCG
CRISPR mutagenesis	FUL1-sgRNA-1	**F:**TGTGGTCTCAATTGTGTTCTCTATTCGCTTCAACGTTTTAGAGC TAGAAATAGCAAG
	FUL1-sgRNA-2	**F:**TGTGGTCTCAATTGACCAGATCGACGTTTCGAGAGTTTTAGAGC TAGAAATAGCAAG
	FUL1-sgRNA-3	**F:**TGTGGTCTCAATTGTGCTTTGTGATGCTGAGGTGTTTTAGAGCT AGAAATAGCAAG

Experiment	Gene	Primer sequence (5'-3')
	FUL1-sgRNA-4	**F:**TGTGGTCTCAATTGCCTACTGATCATACCTCCCGTTTTAGAGCTAGAAATAGCAAG
	FUL2-sgRNA	**F:**TGTGGTCTCAATTGCTTTTTCAAAGAGGCGATCGTTTTAGAGCTAGAAATAGCAAG
	MBP10-sgRNA-1	**F:**TGTGGTCTCAATTGAAAGATGGGGCGGGGTAGGGGTTTTAGAGCTAGAAATAGCAAG
	MBP10-sgRNA-2	**F:**TGTGGTCTCAATTGACATAGCACAGAGATCTCGTGTTTTAGAGCTAGAAATAGCAAG
	MBP10-sgRNA-3	**F:**TGTGGTCTCAATTGATATGAAAATTACTCATACGGTTTTAGAGCTAGAAATAGCAAG
	MBP20-sgRNA-1	**F:**TGTGGTCTCAATTGATAGAGGATGGGAAGAGGTGTTTTAGAGCTAGAAATAGCAAG
	MBP20-sgRNA-2	**F:**TGTGGTCTCAATTGTTGATTGTGTTTTCTACCAAGTTTTAGAGCTAGAAATAGCAAG
	MBP20-sgRNA-3	**F:**TGTGGTCTCAATTGACATTCTCAAAGAGACGATCGTTTTAGAGCTAGAAATAGCAAG
	MBP10/20-sgRNA	**F:**TGTGGTCTCAATTGACATTCTCAAAGAGACGATCGTTTTAGAGCTAGAAATAGCAAG
	CKX6-sgRNA-1	**F:**TGTGGTCTCAATTGTAATGGAATGTCCATGACCTGTTTTAGAGCTAGAAATAGCAAG
	CKX6-sgRNA-2	**F:**TGTGGTCTCAATTGATTATGTCATTAACACAAGAGTTTTAGAGCTAGAAATAGCAAG
	CKX6-sgRNA-3	**F:**TGTGGTCTCAATTGTAAGGAATTGAAGGATTCCAGTTTTAGAGCTAGAAATAGCAAG
	sgRNA universal	**R:** TGTGGTCTCAAGCGTAATGCCAACTTTGTAC
genotyping	*FUL1*	**F:** GACCTTCGCTTATAGCTCTATCCC
		R: CTTCTCCCACATAATGCCTGC
	FUL2	**F:** CTACCTGGGGAGATCCTTCC
		R: TGAGTCCAACTTCAGCATCG
	MBP10	**F:** TGTTGCTCTCCTGCATAGCA
		R: ACAAATTGAAGATGGAGAACGGAG
	MBP20	**F:** CCAATCACAAATCGACAACGCAAC
		R: ATGAGGACATCAATGAGCTGATC
	CKX6	**F:** CTCTTCACCAAAAATCACCTACTTC
		R: CCAACATCCGCGTAAAACCC
	Cas9	**F:** CTTTGGCAATATCGTGGACG
		R: CGTTCTTCTTCTCCCCAGGG
	NPT2	**F:** AGACAATCGGCTGCTCTGAT
		R: AGCCAACGCTATGTCCTGAT
EMSAs	*FUL1-dC*	F: AAAAAGCAGGCTATGGGAAGAGGAAGAGTCCAG
		R: AGAAAGCTGGGTTTACATTAGTACTCTGGTATG
	FUL2	**F:** AAAAAGCAGGCTATGGGTAGAGGAAGAGTACA
		R: AGAAAGCTGGGTTTAACCGTTGAGATGTCGAAGCA
	MBP20	**F:** AAAAAGCAGGCTATGGGAAGAGGTAGGGTAG
		R: AGAAAGCTGGGTTCATCCTTCGTTGCTGACGTGG

Experiment	Gene	Primer sequence (5'-3')
	TM3	**F:** AAAAAGCAGGCTATGGTTCGAGGTAAAACCCAGA
		R: AGAAAGCTGGGTTAGAGACGTCTTTCTCTGCAC
	STM3	**F:** AAAAAGCAGGCTATGGTTCGAGGTAAAACCCAG
		R: AGAAAGCTGGGTTCAAGACCATTCAGGACGCC
	J	**F:** AAAAAGCAGGCTATGGCTAGAGAAAAAATTCAGATC
		R:AGAAAGCTGGGTTTACTTTTTTTTTTCTCCTTCTTCTAATAACAT ACAAAAATAG
	J2	**F:** AAAAAGCAGGCTATGGGAAGAGGAAGAGTAGAAC
		R: AGAAAGCTGGGTTTAGAGCATCCACCCTGGAA
	EJ2	**F:** AAAAAGCAGGCTATGGGAAGAGGAAGAGTTGAGC
		R: AGAAAGCTGGGTTTAAAGCATCCATCCATGAA
	ARR16	**F:** CTCATTCACCTATGATGTAC
		R: GAAAGAATAGAATGTGTATGAAACTG
	mARR16	**F:** TCCCTCCCTCCCTTTCTTGGGG
		R: CCCCAAGAAAGGGAGGGAGGGA
	CKX5	**F:** GTCTGAATATTTTGCATCTATTAC
		R: GCTATCCTCTATAATAACATTTCAC
	mCKX5	**F:** CGATTCCAAGAAAAGCTCG
		R: CGAGCTTTTCTTGGAATCG
	CKX8	**F:** CCAACTAATTAATTACCTAACTAACTAAC
		R: GTTGTCTTCCTTAATGTTAAGATA
	mCKX8	**F:** CTGAACTATTCAAAGAATAC
		R: GTATTCTTTGAATAGTTCAG
	CKX6	**F:** GGAGGGCAAAAAGTTGAAATG
		R: GGGCAAAGATTTGGCTACTT
	mCKX6	**F:** GGCTGCTAAAATCTAAACAAAG
		R: CTTTGTTTAGATTTTAGCAGCC
	FUL1	**F:** CATAGTATAGAAAAAATATCCG
		R: GAAGTTGGTATAATAAAAAATATTAAAAG
	mFUL1	**F:** CTAATGAGGCCTTGTATAGCAC
		R: GTGCTATACAAGGCCTCATTAG

CHAPTER 3

Regulation of reproductive meristem activity by cytokinin oxidases in tomato

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ABSTRACT

The phytohormone cytokinin (CK) positively regulates the activity and function of reproductive meristems, which determine flowering and inflorescence architecture. Cytokinin oxidases/dehydrogenases (*CKXs*) encode enzymes that inactivate CKs to regulate meristem activity and are a genetic target for yield improvement in monocots. Here, we report that CK is increasingly active in the reproductive meristems of tomato, where the *CKX* genes (*SlCKX1-9*) are dynamically expressed. *SlCKX5*, *SlCKX6* and *SlCKX8* are the three *SlCKX* genes that are repressed by FUL2 and MBP20 in the vegetative and transition meristems and may be responsible for increased CK activity in these meristems. We therefore generated single and double CRISPR mutants of these *SlCKX* genes. The three *SlCKX* genes that are repressed by FUL2 and MBP20 in the vegetative and transition meristems. Analysis of these mutants revealed interesting phenotypes in both flowering time and inflorescence branching, where *SlCKX6* alone appeared to be involved in the regulation primary shoot flowering, while all three genes were involved in the control of sympodial shoot flowering time and the regulation of inflorescence branching. Interestingly, this branching appears to be caused by the simultaneous appearance of two IMs on both flanks of the FM, instead of single one in wild-type. Together, our data indicate that CKXs play a prominent role in reproductive meristem development in tomato, which may be further explored to facilitate the genetic enhancement of reproductive production in tomato and other crops.

INTRODUCTION

The shoot apical meristem (SAM) contains pluripotent stem cells and generates primordia that develop into the aerial organs of a plant. In *Arabidopsis*, the SAM generates leaves before transitioning to inflorescence meristem (IM), after which flanking meristems are generated that either develop into flower-bearing shoots or flowers, resulting in an indeterminate inflorescence. Instead of being an indeterminate IM, the vegetative SAM of tomato transitions into a floral meristem (FM) that matures into a flower. However, before maturation, an IM is produced on the flank of the FM, which again produces a new IM before terminating into the next FM. These repeated IM-FM transitions and terminations produce a determinate inflorescence. The vegetative growth is terminated after floral transition in *Arabidopsis*, while that of tomato resumes from the axil of the youngest leaf via a sympodial vegetative meristem that transitions to flowering after producing three leaves, a process that can be repeated indefinitely. The timing of each meristem phase change is vital in the control of plant reproductive programs including flowering,

inflorescence development, and shoot architecture, all of which are of agronomic importance for tomato. For instance, floral transition in sympodial shoots can be accelerated for improved inflorescence compactness, and delayed FM maturation allows additional IMs being formed so that inflorescence branching arises (Pnueli et al., 1998; Lippman et al., 2008a).

The plant hormone cytokinin (CK) has a central function in the regulation of meristem activity. CKs are phytohormones that are involved in many developmental processes in plants, particularly during reproductive development. Upon floral transition, CK increased in the SAM of *Arabidopsis* plants induced by long days (Corbesier et al., 2003). CK deficiency diminished SAM activity, and caused a smaller SAM and late flowering (Werner et al., 2003b; Tokunaga et al., 2012), while elevated cytokinin levels correlated with larger meristems and early flowering (Chaudhury et al., 1993; He and Loh, 2002). It has recently been shown that CK promotes the juvenile-to-adult phase transition via regulation of age pathway components (Werner et al., 2021). After the floral transition, increased CK signaling induces reproductive meristem activity, while decreased signaling retards it, and the dynamic CK signaling subsequently affects inflorescence architecture and thus yield. Indeed, CK has been shown as an evolutionary conserved yield-regulating factor from diverse species including *Arabidopsis* (Bartrina et al., 2011) and rice (Ashikari et al., 2005), despite the divergence of the meristem developmental trajectory to form either a flower or a spikelet.

CK homeostasis in plant cells is tightly regulated by biosynthesis and degradation, of which the latter is catalyzed by cytokinin oxidase/dehydrogenases (CKXs) (Schmülling et al., 2003; Werner et al., 2006). Since the first report of CKX activity, *CKX* genes have been described in a variety of both monocot and dicot species (Pačes et al., 1971). CKX proteins harbor a conserved binding domain for the cofactor flavin adenine dinucleotide (FAD) on their N-termini and small, and highly conserved motifs on the C-termini that may conduct substrate recognition and electron transport (Werner et al., 2003b). These proteins are generally encoded by multigene families. For instance, seven *CKX* genes have been identified in *Arabidopsis* (Schmülling et al., 2003) and 11 in rice (Ashikari et al., 2005). These isoforms vary in their expression patterns, subcellular localization and biochemical properties such as substrate specificity and preferred electron acceptor (Schmülling et al., 2003; Zalabák et al., 2016). The *CKX* genes are expressed in the center of the IM and in the FM in *Arabidopsis*, negatively regulating meristem size and activity (Werner et al., 2003b; Bartrina et al., 2011). Even reduced expression of a single *CKX* gene generally results in increased CK levels, while overexpression causes a decrease (Werner et al., 2003b; Ashikari et al., 2005). Various studies have shown

that the manipulation of CK homeostasis via *CKX* genes impacts inflorescence architecture and yield. Notably, reduced expression of rice *CKX2 (Grain number 1a*) causes CK accumulation in IMs, resulting in panicle branching and enhanced grain production (Ashikari et al., 2005). In tomato, it has recently been shown that there is an increase in CK activity during SAM transition to reproductive development (Steiner et al., 2020; Jiang et al., 2021). Our previous work showed that three tomato *CKX* genes, *SlCKX5*, *SlCKX6* and *SlCKX8* were upregulated in reproductive meristems of *ful1 ful2 mbp10 mbp20* (*slful*) mutants. The *slful* mutants exhibited late flowering, while null mutation of *SlCKX6* caused early flowering (Jiang et al., 2021), indicating a role for CK in reproductive meristems. However, how cytokinin and *CKX* genes affect meristem activity in tomato is far from clear.

In this work, we characterized CK activity in tomato meristems and generated null *slckx* mutants by using CRISPR/Cas9-mutagenesis. Mutant characterization revealed that loss of *CKX* genes causes early flowering in the primary shoot, but late flowering in sympodial shoots. In addition, the *ckx* mutants displayed inflorescence branching, as a result of the simultaneous initiation of two IMs at the flanks of the FM. Together with the observation that CK accumulation increases during the VM to TM transition, these data demonstrate that CK is an important regulator of meristem phase transitions in tomato.

RESULTS

CK signaling is induced in reproductive meristems

To visualize CK signaling during reproductive meristem development, we introduced a synthetic biosensor construct (TCSv2:GUS) into tomato plants and observed an increasing GUS staining during SAM doming (Jiang et al., 2021). Here, we show that the GUS staining was still strong in FM and IM, but later decreases in the FM, being more pronounced in newly developing IMs (Figure 1A). This suggests a role for CK in the regulation of SAM doming and inflorescence development. GUS signal was also observed in the sympodial meristem (SYM) and leaf axillary meristem (AM), suggesting that CK is required for the initiation and growth of these meristems. To determine if the delayed flowering in *slful* mutants could be phenotypically complemented by exogenous CK, we treated the meristems of wild-type (WT) and mutant plants at early VM stage with different concentrations of the synthetic CK 6-benzylaminopurine (BAP). However, earlier flowering was not detected for either genotype (Figure 1B).

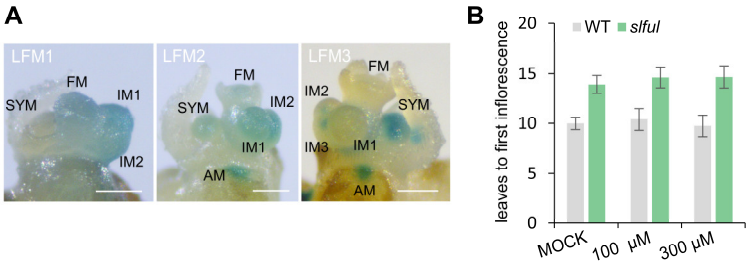
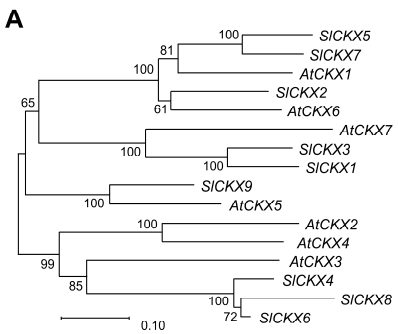


Figure 1. Characterization of CK activity during meristem development. (A) Staining of TCSv2:GUS in meristems at the late inflorescence development stages. LFM1-3: late floral meristem stages 1-3. SYM: sympodial meristem. AM: axillary meristem. White bar: 200 µm. **(B)** Flowering response of WT and *ful1 ful2 mbp10 mbp20* plants upon BAP application. Mean values (± SD) were compared between genotypes using one-way ANOVA followed by a post hoc LSD test.

SlCKX genes display different expression profiles in developing meristems

The tomato genome contains 9 *CKX* genes (*SlCKX1-9*) that are closely related to Arabidopsis *CKXs* (*AtCKX1-7*) (Figure 2A), suggesting that the function of *SlCKXs* may be similar to that of *AtCKXs*. For example, *SlCKX4*, *SlCKX6* and *SlCKX8* are closely related to *AtCKX3*. The high similarity between these three proteins may indicate redundancy, but *CKX* genes are also known for their specific regulation (Werner et al., 2003b; Schwarz et al., 2020), and the temporal and spatial activity of the genes could thus be very different. To understand the putative biological function of *SlCKXs* in reproductive meristems, we analyzed their expression levels in the VM, TM and FM stages by RNA-seq (data Jiang et al., 2021; Figure 2B). *SlCKXs* were expressed lowly, but dynamically, in the meristems, with highest expression levels for *SlCKX1*, *SlCKX8* and *SlCKX9*. Interestingly, *SlCKX1*, *SlCKX5* and *SlCKX9* displayed increased expression levels from VM stage to FM stage, possibly associated with attenuation of cell division in the FM, while those of *SlCKX6* and *SlCKX8* were the opposite, revealing genes that may act in the transition from VM to TM. *SlCKX2*, *SlCKX3* and *SlCKX4* and *SlCKX7* were not, or hardly expressed in the meristems.



B

	VM	TM	FM
SlCKX1	1.54	2.24	3.69
SlCKX2	0.11	0.04	0.01
SlCKX3	0.06	0.07	0.04
SlCKX4	0.00	0.00	0.00
SlCKX5	0.26	0.73	1.00
SlCKX6	1.34	0.91	0.50
SlCKX7	0.00	0.00	0.00
SlCKX8	2.78	1.24	0.69
SlCKX9	0.15	1.20	4.56

Figure 2. Phylogenetic analysis and expression patterns of *SlCKX* genes. **(A)** Phylogenetic tree of CKX proteins in tomato (Sl) and Arabidopsis (At). The phylogenetic tree was constructed using the neighbor-joining method. Bootstrap values estimated with 1000 reiterations were indicated above the branches. **(B)** Normalized gene expression (RPKM) of *SlCKX* genes in the primary shoot meristems of wild-type (data Jiang et al., 2021). The values (mean ± SD) shown are the average of three replicates. VM: vegetative meristem; TM: transition meristem; FM: floral meristem.

Loss of *SlCKX* genes affects flowering and inflorescence complexity

To study the developmental roles of the *SlCKX* genes regulated by *FUL2* and *MBP20* (Chapter 2; Jiang et al., 2021), we generated single and double loss-of-function mutants using CRISPR/Cas9 gene editing. The first exons of *CKX5*, *CKX6* or *CKX8* were targeted by at least two single-guide RNAs. After plant stable transformation, the independent first-generation (T0) lines were analyzed by PCR and sequenced to identify the mutations that caused a frameshift and premature stop codon for each gene (Figure 3A). Because *CKX6* and *CKX8* are highly related to *CKX4*, two of the designed guides were additionally targeting *CKX4*. Checking for mutations in *CKX4* revealed that there were indeed indels present in several of the mutants, but because *CKX4* is not expressed in the meristem at all, we further ignored *CKX4*. T1 progenies with two different homozygous alleles for each genotype were used for further analysis. We quantified flowering time by the number of leaves before the first inflorescence, and only observed a mild but significant acceleration of flowering time in *slckx6* and *slckx6 slckx8* mutant plants compared to WT plants (Figure 3B). However, there was no significant difference of flowering between the two mutants, suggesting that the *SlCKX8* mutation didn't contribute to the phenotype. The observed early flowering may be a result of faster TM doming because of higher CK availability, or the result of an overall larger meristem. To discriminate between these possibilities, we determined TM size for WT and *slckx6* mutant plants (Figure 3C).

We did not observe any size difference between the two genotypes, suggesting that the phenotype is rather due to accelerated doming than to a larger meristem.

The WT plants developed three-leaf sympodial shoots, whereas the *slckx* mutants, except for *slckx5 slclx6*, generated almost one more leaf per shoot, resulting in a lower density of inflorescences compared to WT plants (Figure 3D). Since we observed that the mutant plants produced more branched inflorescences than WT plants (Figure 3E), we determined the inflorescence complexity by quantifying the branching events for the first seven inflorescences of each plant (Figure 3F). All the mutant plants produced more branched inflorescences than the WT, ranging from bi-parous to quadruple-parous inflorescences. Here, the most prominent effect was observed in mutants with *ckx8* knock-out alleles, indicating that *CKX8* does play a distinct role in the IM initiation stage of meristem differentiation.

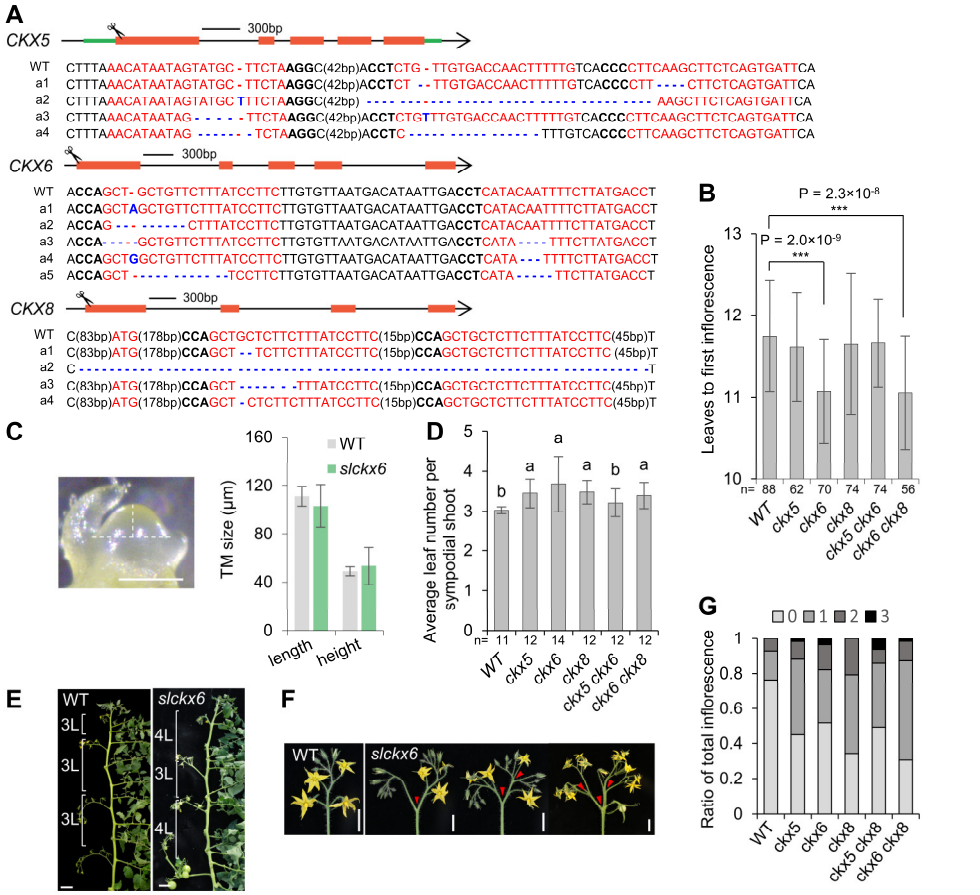


Figure 3. Phenotypic characterization of *SlCKX* mutants. **(A)** Sequences of *SlCKX* mutant alleles (a) generated by CRISPR-Cas9. The following alleles were used in single and double

mutants: *ckx5* (a1, a2), *ckx6* (a1, a2), *ckx8* (a1, a2), *ckx5* (a3, a4)/*ckx6* (a3), *ckx6* (a4, a5)/*ckx8* (a3, a4). The red font highlights sgRNA targets, and black bold indicates protospacer-adjacent motif (PAM) sequences. The red and green boxes indicate exons and UTR of the genes, respectively. Cartoon scissor indicates the targeted exons. **(B)** Primary shoot flowering time measured by number of leaves to the first inflorescence in wild-type and *slckx* mutants. **(C)** Measurement of primary TM size from WT and *slckx6* mutant plants. Dashed lines indicate the width and height used to measure meristem size. White bar: 200μm. **(D)** Sympodial flowering times of the same set of plants as in (B). The average leaf number of all sympodial shoots from each plant was used for statistical significance analysis. **(E)** Representative sympodial shoots from WT and *slckx* mutant plants. L: leaf; Scale bars: 5 cm. in B and D, n indicates numbers of individual plants. In B, C, D, mean values (± SD) were analyzed by one-way ANOVA followed by post hoc LSD tests, different letters indicate the difference at $P<0.05$ level in D. **(F)** Representative inflorescences of WT and *slckx* mutants. Red arrowheads indicate branching events. Scale bars: 2 cm. **(G)** Proportion of branched inflorescences in the indicated genotypes.

Inflorescence branching is induced by loss of *CKX5, CKX6* and *CKX8*

Inflorescence branching arises when additional IMs are formed before FM maturation. To investigate whether the inflorescence branching resulted from delayed FM maturation, we examined the meristem growth dynamics of inflorescence development in detail in the *slckx6* mutant (Figure 4). Interestingly, we observed that two IMs were simultaneously formed on both sides of the *slckx6* FM, which is different from the situation in the *ful2 mbp20* mutant, where delayed maturation of the FM results in the formation of an additional IM that emerges from the side-front. In contrast to the second IM in *ful2 mbp20* mutants, the two IMs of the *slckx6* mutant appear to be at the same developmental stage after arising, indicating that they emerge at the same time.

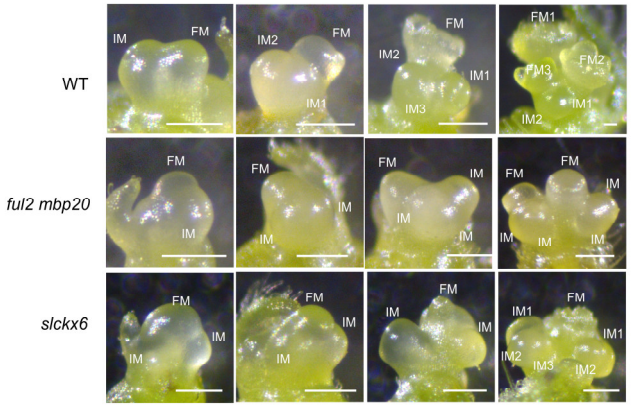


Figure 4. Meristem characterization during inflorescence development. A developmental series of meristems of WT, *ful2 mbp20*, and the *slckx6* mutant from the FM stage to an inflorescence. FM: floral meristem; IM: inflorescence meristem. White bar: 200 µm.

DISCUSSION

In most plants, the floral transition is coupled with SAM doming, which is a dramatic change triggered by many factors such as hormones, microRNAs, sugars, and small proteins. Of the hormones, CK is a prominent inducer of cell proliferation (Miller et al., 1955), and can probably positively regulate cell division during SAM transition to allow doming of the meristem. In line with this hypothesis, reduced CK levels may inhibit SAM doming and thereby delay flowering. In *Arabidopsis*, CK deficiency through overexpression of *CKXs* diminishes SAM activity and indeed retards flowering (Werner et al., 2003b). We show here that the tomato *SlCKX6* gene is also repressing flowering, and that its mutation results in early flowering, probably due to faster accumulation of CK in the meristem. The observed acceleration was mild, which can be explained by the role of the *SlCKX*s downstream of key flowering regulators. Additionally, other *SlCKX*s may have a partial redundant function and may increase the mutant phenotype when mutated in the *ckx6* mutant background. Surprisingly, the expression of *SlCKX8* is higher than that of *SlCKX6* in meristems, but its mutation did not cause a flowering time phenotype (Figure 2B, Figure 3B). This may be explained by a difference in expression pattern within the tissue, a different subcellular localization or diverged biochemical properties. For example, *SlCKX8* may simply be expressed throughout all sampled cells, while *SlCKX6* may be specifically expressed. Both subcellular localization and substrate specificity may also play a role, as specific CK metabolite spectra occur in different subcellular compartments, as well as extracellularly (Faiss et al., 1997; Benková et al., 1999), corresponding to different enzymatic activity and subcellular localizations of the Arabidopsis CKX proteins (Werner et al., 2003b; Skalický et al., 2018). For instance, the AtCKX1 enzymatic activity is greater under acidic conditions in vitro, which is in line with its vacuolar localization (Werner et al., 2003b). However, the elucidation of the subcellular targeting and/or activity for the *SlCKXs* requires additional experiments before we can understand the differences in flowering time phenotype between the *slckx6* and *slckx8* mutants. CK signalling needs to be dynamic during inflorescence development, as activity needs to be induced to get TM doming, while reduced from TM to FM, where cell division rate needs to slow down to get differentiation of the FM. *SlCKXs* are always performing the same role (repressing meristem activity), but their regulation differs to get different effects in the different meristems. For instance, *SlCKX1* and *SlCKX9* are highly expressed in FM, implying

a repressing function on FM size. Loss of both genes may elevate CK levels and cause large FM, possibly leading to the formation of additional organs.

Although the floral transition in the primary shoot is slower than that in the sympodial shoot, the flowering behaviour is the same in both shoots. Interestingly, we observed a mild early floral transition in primary shoots, but late flowering in sympodial shoots of *slckx6* mutant plants. This suggests that *SlCKX6* functions in a different manner in both meristems. Also, mutations of *SlCKX5* and *SlCKX8* only affected sympodial flowering, further suggesting that *SlCKX* genes play a bigger role in sympodial shoot meristems than in primary shoot meristems, although the observed delay was only mild. Since we determined flowering time by the number of leaves until meristem transition, it is possible that the enhanced CK signalling confined to the late primary shoot VM may have enhanced meristem doming and thereby flowering, while more general enhanced CK signalling in the sympodial VM may have resulted in the formation of more leaves before the flowering transition. In the *slckx5 slckx6* double mutants, we did not observe flowering phenotype except for inflorescence branching, nor did we find enhancement of the phenotype in the *slckx6 slckx8* mutants compared to the single mutants. This may be due to the low number of plants that was phenotyped in combination with the variation observed, because four out of 12 *slckx5 slcxk6* plants showed clear late flowering in sympodial shoots. Together, more in depth research about meristem size and frequency of primordium formation needs to be combined with detailed expression data for *SlCKXs* to obtain more insight into the exact cause of the observed phenotypes.

Reproductive meristem activity is crucial for the shaping of inflorescence architecture. In chapter 2 (Jiang et al., 2021), we showed that the inflorescence branching of *ful1 ful2 mbp20 mbp10* mutants is caused by delayed maturation of the FM, which occasionally allows a 2nd IM to form. *SlCKX5*, *SlCKX6* and *SlCKX8* were not differentially expressed in the FM/IMs of *slful* quadruple mutants and therefore probably not responsible for the increased branching phenotype in the *slful* quadruple mutant. However, we did identify increased inflorescence branching in all *sl*ckx mutants, but detailed analysis revealed that this was not caused by the same process as in the *slful* quadruple mutant. Interestingly, we show that the additional IMs grow symmetrically on the FM flanks (Figure 4A), instead of from the front-side as report previously (Lippman et al., 2008a; Park et al., 2012), and they arise simultaneously, instead of sequentially as is the case in the mutants that exhibit delayed FM maturation, such as *s* and the *slful* quadruple mutant. To elucidate the cause of this simultaneous initiation, we will have to perform additional experiments, such as introduction of the TCSv2:GUS reporter in the *ckx* mutant background and careful measuring of meristem size. A bigger FM meristem in the *slckx6* mutant could

explain the initiation of additional meristems/primordia, similar to the situation in Arabidopsis, where loss of *CKX3*/*CKX5* increased the IM size and thereby lead to more flower primordia (Bartrina et al., 2011). However, we didn't observe clear differences in meristem size between WT and *slckx6* (Figure 3C), possibly because the discrepancy is too small to be measured, which can also explain why the *slckx6* mutant meristem can only initiate one additional IM. The meristem size difference may be more prominent in higher order mutants of *SlCKXs* and it would be interesting to explore how key meristematic genes, such as *WUSCHEL* and *CLAVATA* (Clark et al., 1995; Laux et al., 1996; Schoof et al., 2000), genetically interact with the *SlCKXs*. In conclusion, we demonstrated that *SlCKXs* are associated with tomato reproductive development by regulation of meristem activity.

Yield enhancement is an important agronomical goal of breeding for most crop species. CK has been implicated in various yield-determining traits, particularly in inflorescence structure, which was first noted during the analysis of the *OsCKX2* gene in rice (Ashikari et al., 2005; Han et al., 2014a; Jameson and Song, 2016). Since then, *CKX* genes have been a target for yield improvement in cereals, but much less is known about their role in dicot plants. It was shown that simultaneous mutation of several *CKX* genes in the closely related species Arabidopsis and Brassica caused more flowers and higher yield (Bartrina et al., 2011; Schwarz et al., 2020), suggesting similar functional roles of the orthologous *CKX* genes in monocots and dicots, despite the fundamental differences in inflorescence development. In this work, the branched inflorescence caused by *SlCKX6* mutation may produce more flowers and thus improve the fruit yield. Our results extend the concept of CK as a yield-regulating factor, and it would be very plausible to achieve productivity enhancement in closely related crop species such as pepper.

MATERIALS AND METHODS

Plant materials and growing conditions

Seeds of Tomato cv. Moneyberg were germinated either on ½ MS for tissue culture transformation or on moistened filter paper for genotyping. Seedlings were transferred to rockwool and cultivated in a 21 °C growth chamber (16h light/8h dark) for four to six weeks. Finally, plants were transferred to the greenhouse with ambient temperature (>20 °C) under natural light supplemented with artificial sodium lights.

CRISPR construct generation and stable tomato transformation

The constructs to create mutations in the *SlCKX* genes using CRISPR/Cas9 was generated using GoldenGate cloning and the MoClo toolkit according to (Weber et al., 2011). In short, the online program http://www.rgenome.net/cas-designer/ (Park et al., 2015) was used for sgRNA designing. Three sgRNAs were used to target each gene, each sgRNA was fused to the synthetic U6 promoter U6p::sgRNA and cut-ligated to level 1 vectors. For constructs with three guides, the level 1 constructs pICH47732-NOSpro::NPTII::OCST, pICH47742-35S::Cas9::NOST, pICH47751-35S::GFP::ter35S, pICH47761-sgRNA1, pICH47772-sgRNA2, pICH47781-sgRNA3, and the linker pICH41822-pELE6 were cut-ligated into the level 2 vector pICSL4723 as described. For the constructs with six guides, a two-step Golden Gate cut-ligation was performed. The pICH47732-NOSpro::NPTII::OCST, pICH47742-35S::Cas9::NOST, pICH47751-35S::GFP::ter35S, the first two guides pICH47761-sgRNA1 and pICH47772-sgRNA1, and linker pICH49266-pELB2 were cut-ligated with pICSL4723 to create an intermediate level2 (pL2i-1). This vector was then used to construct the final level2 (pL2-2) by cut-ligating it with pICH47781-sgRNA3, pICH47791-sgRNA4, pICH47732-sgRNA5, pICH47742-sgRNA6 and linker pICH41744-pELB2. The level 2 constructs were transformed into *Agrobacterium tumefaciens* C58C1 for plant stable transformation (Van Roekel et al., 1993). All primers are listed in Supplemental Table 1.

Genotyping

The targeted regions were amplified by a PCR conducted directly on sampled leaf tissue by using the Phire Plant Direct PCR kit (Thermo Scientific), and then sequenced for mutations. After the Cas9 construct was segregated out, plants with homozygous or biallelic mutations were grown for further study. Primers used in genotyping are listed in Supplemental Table 1.

GUS Histochemistry

Histochemical analysis of the TCSv2::GUS reporter was performed according to (Steiner et al., 2016) Plant tissue was vacuum infiltrated for 5 min in a solution containing 2.0 mM potassium ferri- and ferrocyanide, and incubated overnight at 37 °C. Tissue was then cleared in 70% ethanol prior to imaging.

Meristem imaging and size measurement

Young plants were used to dissect shoot apices by forceps, and older leaf primordia were removed to expose the meristems under the stereomicro-scope (Stemi 508,

Zeiss) with a coupled camera (AxioCam IC, Zeiss Germany). Live meristems were imaged immediately after dissection. The SAM size was measured at the maximum width between leaf primordia using Leica Application Suite v4.9 software.

BAP treatment

For cytokinin treatment, the SAM at one-leaf before doming stage was supplied with one drop of 6-benzylaminopurine (Sigma Aldrich) of different concentrations containing 0.05% Tween 20. The treatment was once every two days until floral transition.

Accession numbers

SlCKX1, Solyc08g061930; *SlCKX2*, Solyc01g088160; *SlCKX3*, Solyc08g061920; *SlCKX4*, Solyc12g008920, *SlCKX5*, Solyc04g016430; *SlCKX6*, Solyc12g008900; *SlCKX7*, Solyc10g079870; *SlCKX8*, Solyc10g017990; *SlCKX9*, Solyc04g080820.

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AUTHOR CONTRIBUTIONS

M.B. and X.J. conceived the project and designed the experiments; X.J. performed the tomato CRISPR/cas9 mutagenesis, meristem stereomicroscope examination and imaging, and qPCRs; M.B. and X.J. analyzed the data and prepared the figures; G.C.A., M.B. and X.J. wrote the article.

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Supplemental Table1. Primer used in this study

Experiment	Gene	Primer sequence (5'-3')
CRISPR mutagenesis	CKX5-sgRNA-1	**F:**TGTGGTCTCAATTGAACATAATAGTATGCTTCTAGTTTTAGAGCTAGAAATAGCAAG
	CKX5-sgRNA-2	**F:**TGTGGTCTCAATTGCAAAAAGTTGGTCACAACAGGTTTTAGAGCTAGAAATAGCAAG
	CKX5-sgRNA-3	**F:**TGTGGTCTCAATTGAATCACTGAGAAGCTTGAAGGTTTTAGAGCTAGAAATAGCAAG
	CKX6-sgRNA-1	**F:**TGTGGTCTCAATTGTAATGGAATGTCCATGACCTGTTTTAGAGCTAGAAATAGCAAG
	CKX6-sgRNA-2	**F:**TGTGGTCTCAATTGATTATGTCATTAACACAAGAGTTTTAGAGCTAGAAATAGCAAG
	CKX6-sgRNA-3	**F:**TGTGGTCTCAATTGTAAGGAATTGAAGGATTCCAGTTTTAGAGCTAGAAATAGCAAG
	CKX8-sgRNA-1	**F:**TGTGGTCTCAATTGAAAGGAATTGAAGGATTCCAGTTTTAGAGCTAGAAATAGCAAG
	CKX8-sgRNA-2	**F:**TGTGGTCTCAATTGAATGATCTTTCCAAAATCTTGTTTTAGAGCTAGAAATAGCAAG
	CKX8-sgRNA-3	**F:**TGTGGTCTCAATTGCAATATGAAAAGGGATAGAAGTTTTAGAGCTAGAAATAGCAAG
	sgRNA	**R:** TGTGGTCTCAAGCGTAATGCCAACTTTGTAC
genotyping	*CKX5*	**F:** TTCTTGAGGCCATTCTACACC
		R: CCATGTTTGAATGCTTGCCCA
	CKX6	**F:** CTCTTCACCAAAAATCACCTACTTC
		R: CCAACATCCGCGTAAAACCC
	CKX8	**F:** TCACTCACTTTTCTCTTCACCA
		R: CATGAACGTTACTTATTTGAGGACC
	Cas9	**F:** CTTTGGCAATATCGTGGACG
		R: CGTTCTTCTTCTCCCCAGGG
	NPT2	**F:** AGACAATCGGCTGCTCTGAT
		R: AGCCAACGCTATGTCCTGAT

CHAPTER 4

Reproductive meristem identity in tomato is specified by overlapping functions of *AP1/FUL*-like genes

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ABSTRACT

The tomato inflorescence architecture is determined by the activities of meristems after floral transition, which involves the floral meristem (FM), and the inflorescence meristem (IM) formed on the flank of the developing FM. The newly formed IM also develops into an FM, resulting in sequential FM-IM maturations, which generate a zigzagged inflorescence with multiple flowers. Both the timing of FM specification and the subsequent onset of IM-identity genes in the newly formed flanking meristem are important processes in the development of inflorescences. Previous work showed that loss of the tomato *FRUITFULL*-like genes *FUL2* and *MBP20* delays the floral transition and FM maturation, allowing additional flanking meristems to be formed with an inflorescence meristem (IM) fate, while subsequent flower formation is unaffected, resulting in a branched inflorescence. Interestingly, the flanking meristem adopts a vegetative fate in mutants of the *FUL2/MBP20* homolog *MACROCALYX* (*MC*) (ortholog of Arabidopsis *APETALA 1 (AP1)*). Here, we show that combined mutations of the three genes cause delayed FM maturation that allows additional formation of flanking meristems, which adopt a vegetative fate instead of IM fate, resulting in a massively bushy inflorescence with severely reduced fruit productivity. Moreover, in *mc ful2 mbp20* triple mutants, sympodial shoot flowering was massively delayed, leading to a highly vegetative shoot. Gene expression analysis in the sympodial vegetative meristem revealed that this phenotype is not caused by upregulation of the well-known repressors *SP* and *SP5G*, but that several *CKX* genes are highly upregulated in the triple mutant, consistent with their upregulation in *ful2 mbp20* primary vegetative meristems. FUL2, MBP20 and MC may thus regulate both primary shoot and sympodial shoot flowering through control of CK homeostasis. Taken together, we demonstrate that *FUL2/MBP20* and *MC* play overlapping roles in both the timing of the floral transition and the specification of FM/IM identity, with a more prominent role for *FUL2/MBP20* in the first process and for *MC* in the latter process.

INTRODUCTION

The timing of meristem phase changes controls plant agricultural traits such as flowering time and inflorescence architecture and is therefore finely tuned by gene regulatory networks (Kwiatkowska, 2008; Park et al., 2012; Yoshida et al., 2013). In tomato, the phase change of the transition to flowering is characterized by shoot apical meristem (SAM) doming, when the flat vegetative meristem (VM) develops a domed shape, resulting in the transition meristem (TM) and terminating vegetative growth of the primary shoot. Then, the TM acquires floral meristem (FM) fate, and

before it initiates floral organs, a new meristem arises on its flank, which adopts inflorescence meristem (IM) fate before maturing into an FM and terminating into a flower (Lippman et al., 2008a). Thus, the IM follows the same developmental trajectory as the TM, resulting in sequential FM-IM maturations, which generate a zigzagged inflorescence with multiple flowers. Therefore, the interactions between FM and IM, and the timing of their formation and maturation, are crucial in shaping the tomato inflorescence.

The specification of FM fate in *Arabidopsis* is mainly mediated by *LEAFY* (*LFY*) and *APETALA1* (*AP1*), which play dual roles in determining FM identity and floral organ patterning (Mandel et al., 1992; Weigel et al., 1992; Parcy et al., 1998). Functional conservation of both genes has been shown in controlling inflorescence architecture in tomato, where loss of *FALSIFLORA* (*FA*, homolog of *LFY*) results in failure to establish the FM, leading to extremely branched inflorescences composed of massive vegetative meristems (Allen and Sussex, 1996; Molinero-Rosales et al., 1999). FA and its transcription factor partner, ANANTHA (AN*,* homolog of UFO), together specify FM identity. The inflorescences of *an* mutants cannot specify FMs, but only generate new IMs, making a cauliflower-like structure (Allen and Sussex, 1996; Lippman et al., 2008a). Moreover, delayed expression of *AN* causes delayed FM maturation, generating a highly branched inflorescence in *compound inflorescence* (*s,* homolog of *WOX9*) mutants (Lippman et al., 2008a), whereas precocious activation of *FA* and *AN* result in a solitary-flower inflorescence in the *terminating flower* (*tmf*) mutant (MacAlister et al., 2012a; Soyk et al., 2017a). While the specification of the first primary shoot FM appears to depend solely on FA and AN, successive FMs develop from the flanking IMs, and thus also depend on correct specification of IM identity. These IMs could be reverted to the vegetative state instead of adopting an FM fate, such as in *macrocalyx* (*MC*, homolog of *AP1*) mutants, of which inflorescences revert to vegetative growth after producing a few fertile flowers with long leafy-like sepals (Nakano et al., 2012; Yuste-Lisbona et al., 2016b). Likewise, loss-of-function of *SINGLE FLOWER TRUSS* (*SFT*, homolog of *FT*) or *JOINTLESS* (*J*, homolog of *SVP*) also leads to inflorescence vegetative reversion without disturbing the sympodial growth habit of the plants (Molinero-Rosales et al., 2004; Szymkowiak and Irish, 2006). The fact that inflorescence vegetative reversion in *mc sft* and *mc j* double mutants occurred earlier than in single mutants indicates that these three genes synergistically interact to specify FM identity and/or confer IM identity in tomato (Thouet et al., 2012a; Yuste-Lisbona et al., 2016b). Despite many mutant studies, the regulatory network of FM and IM specification in tomato is still fragmented due to the lack of gene interaction studies.

To date, several other genes controlling inflorescence development through the control of meristem fate change have been reported in tomato, including *FRUITFULL*(*FUL*)-like genes (Jiang et al., 2021). *FUL* encodes a MADS-domain transcription factor from the angiosperm-specific *AP1/FUL* subfamily, which underwent multiple duplications throughout angiosperm diversification resulting in the eudicot *AP1* (*euAP1*)-, *euFULI*- and *euFULII* clades (Litt and Irish, 2003). In Arabidopsis, *AP1* is required for specification of FM identity, together with its paralog *CAULIFLOWER* (*CAL*), and for sepal and petal development. In the *ap1* mutant, sepals and petals are transformed into bract-like organs, from which secondary flowers are formed (Irish and Sussex, 1990; Bowman et al., 1993). *ful* single mutant plants display late flowering, wider cauline leaves, and defective fruit development, but develop normal flowers (Gu et al., 1998; Ferrándiz et al., 2000; Melzer et al., 2008b; Bemer et al., 2017). The late flowering phenotype of the *ful* mutant, which is enhanced when also the homologous gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* is mutated, indicates that *FUL* plays a role in the transition from VM to IM in *Arabidopsis*. Additionally, *FUL* contributes to FM specification, especially when *AP1* is absent. *FUL* is ectopically expressed in the FM when *AP1* is depleted, and further loss of FM identity is observed when *FUL* is depleted in the *ap1* mutant, suggesting *FUL*'s capability of specifying FM identity (Mandel and Yanofsky, 1995; Ferrándiz et al., 2000). However, the meristems that are formed in the triple *ap1 cal ful* mutant after the floral transition produce more vegetative-like organs than the meristems in the *ap1 cal* double mutant, probably because inflorescence fate is more lost due to a lower LFY:TFL1 ratio in the triple mutant (Ferrándiz et al., 2000). Thus, in Arabidopsis, *AP1* and *CAL* are characterized as FM identity genes, while *FUL* additionally contributes to IM identity (Gu et al., 1998; Ferrándiz et al., 2000; Becker and Theißen, 2003). In diverse other species, including rice (Kobayashi et al., 2012), wheat (Kinjo et al., 2012), panax ginseng (Ahn et al., 2015), poppy (Pabón-Mora et al., 2012), aquilegia (Pabón-Mora et al., 2013), and petunia (Morel et al., 2019a), *AP1*- and *FUL*-like genes also function in inflorescence development by regulating IM- and/or FM-specification. The tomato genome carries four *FUL*-like genes (*FUL1, FUL2, MBP10, MBP20*), among which the *euFULI*-clade genes *FUL1/2* are well known as key regulators of fruit development (Bemer et al., 2012; Shima et al., 2013; Wang et al., 2019a). Additionally, we showed that mutations in *FUL1/2* and the *euFULII*-clade gene *MBP20*, individually or combined, result in late flowering and increased branching by delaying SAM doming and FM maturation, respectively (Chapter 2;(Jiang et al., 2021). *MC,* on the other hand, has an important role in tomato FM specification, as described above, and the *mc* mutant additionally exhibits leaf-like sepals and a mild delay in flowering time (Yuste-Lisbona et al., 2016b). However, it is unclear to what

extent the functions of *MC* and the *FUL*-like genes are overlapping, and, most importantly, how these closely related genes together regulate meristem phase change and meristem identity specification during reproductive development in tomato.

In this study, we investigated the functional divergence of *MC* and *SlFUL* by comparing their expression profiles and protein-protein interaction patterns and by analysing their *in vivo* functions with CRISPR-induced mutagenesis. We discovered that the combined mutations of *MC* and *SlFUL* cause enhanced delayed flowering and inflorescence defects, indicating that they have overlapping functions in promotion of flowering and conferring IM identity, although the contribution of the genes to these functions differs. The severely late sympodial flowering in the *mc ful2 mbp20* triple mutant is possibly due to delayed CK accumulation due to high expression of *SlCKXs.*

RESULTS

Tomato *AP1*/*FUL*-like genes are expressed dynamically during meristem maturation

Since the *mc* mutant displays mildly delayed flowering and frequent reversion of the inflorescence to vegetative growth (Molinero-Rosales et al., 2004; Szymkowiak and Irish, 2006), we investigated its expression during SAM maturation in the transcriptome data from (Jiang et al., 2021)(Figure 1A). *MC* is expressed lower than *FUL2* and *MBP20* in the VM, at a similar level as *FUL1*, but its expression sharply increases in TM and FM, reaching a much higher level than any of the *SlFULs*. This expression pattern is consistent with its key role in FM development, and its mild flowering time phenotype.

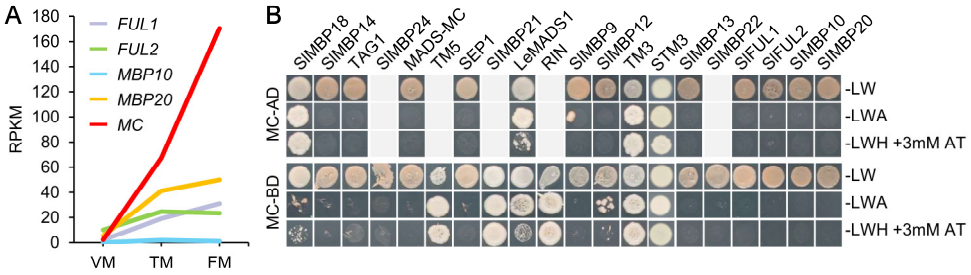

Figure 1. **Characterization of the Tomato *AP1*/*FUL* MADS-Box genes/proteins.** (A) Normalized gene expression (RPKM) of *AP1/FUL*-like genes in the primary shoot meristem of wild-type. The values (mean ± SD) shown are the average of three replicates. VM:

vegetative meristem; TM: transition meristem; FM: floral meristem. (B) Protein interactions between the MC protein and other tomato MADS-domain proteins in a yeast-2-hybrid assay. Grey boxes indicate that the interaction was not tested, in most cases, because the bait gave auto-activation. L, leucine; W, tryptophan; H, histidine; A, adenine; 3-AT, 3-amino-1,2,4-triazole.

FUL2 and MC display a similar protein-protein interaction pattern

To investigate the hypothesis that MC and the SlFULs may act redundantly in the same protein complexes, we tested the protein-protein interactions that could be formed by MC and compared them with the set that we identified before for the four FUL-like proteins (see Chapter 2; Jiang et al., 2021). Among the FUL-like proteins, FUL2 displayed the most extensive pattern, interacting with ten of the 21 tested tomato MADS-domain proteins, while FUL1 and MBP20 interacted with a subset of these, and MBP10 hardly interacted at all (Jiang et al., 2021). We used the same set of MADS-domain proteins to screen for the MC protein-protein interactions and found that MC could interact with the same proteins as FUL2, except for TAG1, for which no interaction was observed (Figure 1B) (Leseberg et al., 2008; Jiang et al., 2021). Like the SlFULs, MC cannot form a homodimer nor can it heterodimerize with any of the SlFULs. Thus, MC may have similar biological roles to the SlFULs.

Tomato *AP1/FUL*-like genes additively promote flowering

To investigate the unique and redundant functions of *MC* and the *SlFUL*s *in planta*, we knocked out *MC* in the wild-type (WT) as well as in the *ful2 mbp20* mutant (Jiang et al., 2021), to generate single, double and triple null mutants by CRISPR-cas9 mutagenesis. Because *MBP10* and *FUL1* do not, or only mildly, contribute to the flowering phenotypes (Jiang et al., 2021), we left them out of the analysis. We targeted the first exon of *MC* by three single-guide RNAs and obtained multiple independent first-generation (T0) diploid lines with homozygous or biallelic *MC* mutations in each background (Figure 2A). To obtain homozygous or biallelic stable mutants, we sequenced T1 progeny to isolate two out-of-frame alleles that caused premature stop codons. The performance of all CRISPR mutants was thoroughly analyzed by detailed phenotyping of T1 plants. We confirmed the delayed flowering in primary shoots of the *mc* mutant, which averaged one additional leaf compared to WT plants.

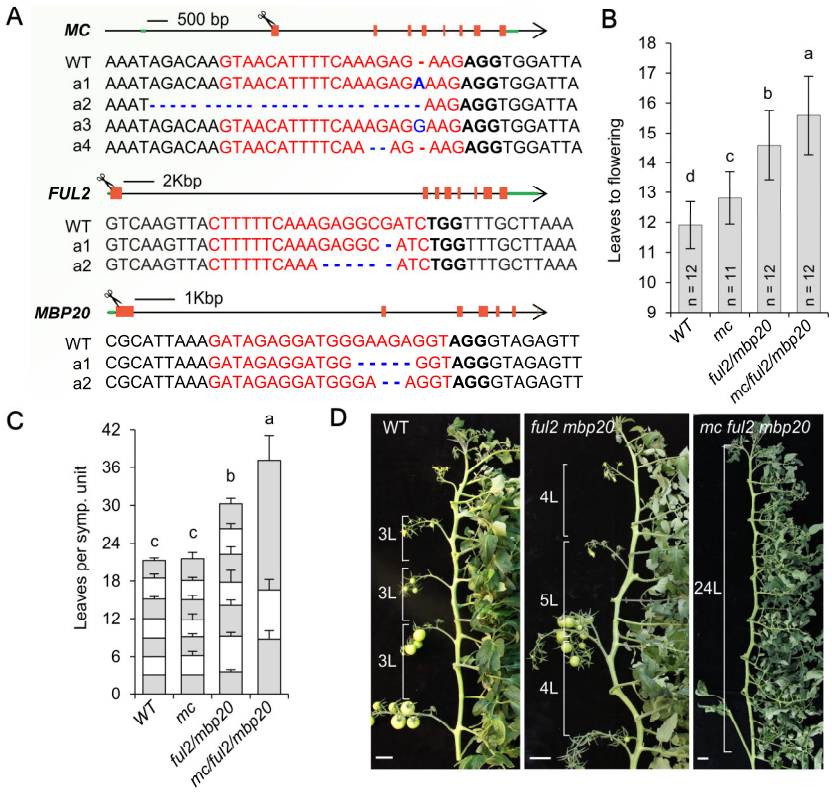

Figure 2. Multigene knockouts of tomato *AP1*/*FUL*-like genes display delayed flowering. **(A)** sequences from T1 genotyping showing CRISPR-induced out-of-frame deletions and insertions (in blue) for *MC*, *FUL2* and *MBP20*. For *MC*, allele 1 (a1) and a2 are from the two *mc* single mutant lines, and a3, a4 from the *mc ful2 mbp20* mutant lines. The red font highlights sgRNA targets, and black bold indicates protospacer-adjacent motif (PAM) sequences. The red boxes indicate coding exons, and the UTRs of the genes are indicated in green. Cartoon scissors indicate the targeted exons. **(B)** Primary shoot flowering time indicated by the number of leaves to the first inflorescence in wild-type and mutants. n: numbers of individual plants measured. **(C)** Flowering times from the successive sympodial shoots of the same set of plants as in (**B**). The average leaf number of all sympodial shoots from each plant was used for statistical significance analysis. **(D)** Representative main shoots from wild-type and mutant plants. For the *mc ful2 mbp20* mutant plant, only the third sympodial unit is shown, and the third inflorescence was removed due to bushy vegetative growth. L: leaf; Scale bars: 5 cm. In B and C, mean values (± SD) were compared between genotypes using one-way ANOVA followed by a post hoc LSD test, different letters indicate the difference at P<0.05 level.

We also observed that the *MC* mutation enhanced the *ful2 mbp20* phenotype in primary shoots (Figure 2B), but the *ful2 mbp20* double mutant exhibited more delayed flowering than the *mc* mutant. In sympodial shoots, flowering of the *mc*

mutant was unaffected, developing three-leaf sympodial shoots as in WT plants (Figure 2 C, D). However, the combined loss of *MC, FUL2* and *MBP20* dramatically delayed sympodial flowering, causing shoots to remain in a vegetative state progressively longer, with even a 24-leaf floral transition in the third sympodial shoot. After the 3rd sympodial shoot, the plants did not flower anymore. These results suggest that *FUL2/MBP20* are most important for flowering time in both the primary and sympodial shoots, but that *MC* plays a redundant role in the sympodial shoot as well, where the *AP1/FUL*-like genes appear major determinants of the floral transition.

Gene expression in VM of sympodial shoot

Since the sympodial shoot flowering is unaffected in *mc* mutants and mildly delayed in *ful2 mbp20* mutants, but dramatically enhanced in *mc ful2 mbp20* mutant, we wondered how the three genes together regulate sympodial flowering time. It is well-known that tomato sympodial cycling is maintained by a balance between flower-promoting and flower-repressing signals, where the former is mainly represented by the 'florigen' *SINGLE FLOWER TRUSS (SFT)*, and the latter by *SELF-PRUNING* (*SP*, homolog of *TFL1*) and *SP-interacting G-BOX* (*SPGB*, homolog of *FD*) (Carmel-Goren et al., 2003; Shalit et al., 2009; Park et al., 2014) If *SP* or *SPBG* would be up-regulated in the triple mutant, this could well explain the observed severe sympodial flowering time phenotype. Alternatively, release of suppression of the *SlCKX5, SlCKX6* and *SlCKX8* genes in the *mc ful2 mbp20* mutant could lead to delayed floral transition in the SYM, similar to the regulation of CK homeostasis by FUL2 and MBP20 in the primary meristem (Jiang et al., 2021). To investigate these possibilities, we determined the expression profiles of these genes in sympodial VM (SVM) of WT, *ful2 mbp20* and the triple mutant plants (Figure 3A). Surprisingly, in SVM, *MC* had the highest expression level, while *FUL2* had the lowest (Figure 3B), which is the opposite pattern of that in the primary VM (Figure 1A). *SP, SPGB* and the three *SlCKXs* were expressed at the same level in SVM of WT and *ful2 mbp20*. However, in the triple mutants, SP and SPGB expression were slightly decreased while that of *SlCKXs* was considerably increased (Figure 3C), indicating that AP1/FUL-like genes may also mediate sympodial shoot flowering via CK homeostasis.

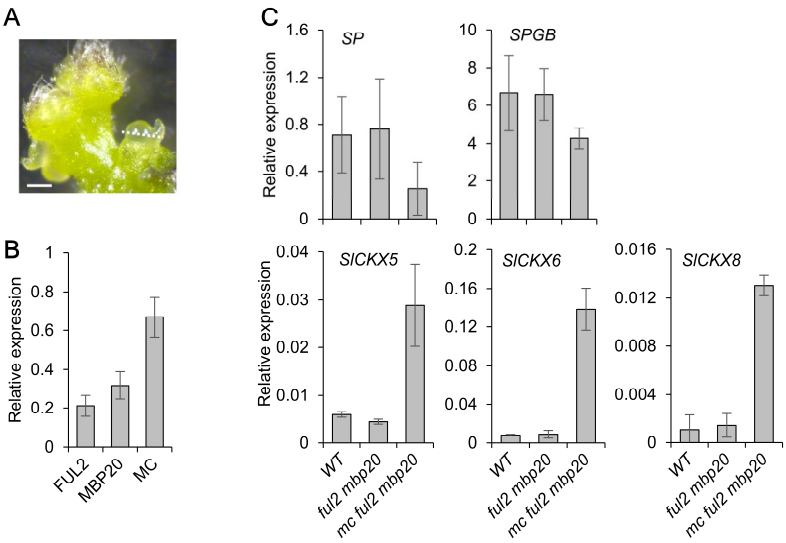

Figure 3. Gene expression analysis in sympodial vegetative meristems (SVM). (A) Manual microdissection of the vegetative meristem of the first sympodial shoot. The white dashed line indicates the dissected tissue. White bar: 200 µm. **(B) and (C)** Gene expression obtained by qRT-PCR. Three biological replicates of WT and *ful2 mbp20*, and two of *mc ful2 mbp20* were used. The error bars represent SD of the mean.

4

Higher-order *ap1 ful* mutants show enhanced inflorescence defects

It has previously been shown that the inflorescences of *mc* plants revert to vegetative growth after a few flowers with leaf-like sepals have been formed (Nakano et al., 2012; Yuste-Lisbona et al., 2016b). We observed the same in our *mc* single mutants but found that *FUL2* and *MBP20* mutations severely enhanced the vegetative reversion of the *mc* mutant inflorescences. The triple mutants almost completely reverted to shoot growth, bearing only very few flowers with leaf-like sepals, which are capable of setting fruit (Figure 4A, Supplementary Figure 1). To explore the developmental basis for the inflorescence defects in each genotype, we dissected and compared the SAM growth dynamics at different reproductive stages. As previously shown, the maturing FM gives rise to a lateral meristem that adopts IM fate to mature into an FM in WT, and delayed FM maturation allows more IMs being formed in *ful2 mbp20* mutants, so that inflorescence branching occurs (Figure 4B). In *mc*, after initiation of a few flowers, the lateral meristem adopts a vegetative fate of either leaf development or shoot growth, and this vegetative reversion occurs much earlier in *mc ful2 mbp20,* initiating only one FM that develops into a flower with leaf-like sepals (Figure 4B, Supplementary Figure 1). The maturation of the FM in

the triple mutant is delayed as is that in *ful2 mbp20* mutants, which allows a few IM-like (IML) meristems to be formed on its flank and these IMLs adopt vegetative fate to make a bushy inflorescence. These results suggest that *MC* plays a major role in specifying IM fate with a minor contribution from *FUL2/MBP20*. This is supported by the fact that the inflorescences of *slful* higher order mutants revert to leaf and/or shoot growth occasionally, but more often than those of WT plants (Supplemental Figure 2)

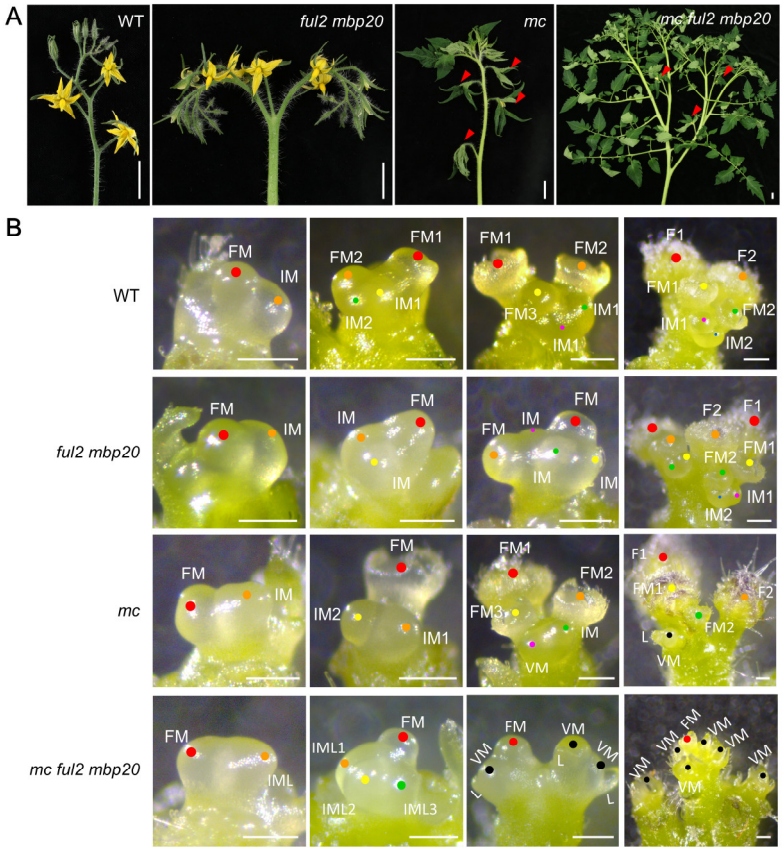

Figure 4. Loss of *AP1*/*FUL*-like genes causes severe inflorescence defects by changing IM fate. **(A)** representative inflorescences from WT, *mc*, *ful2 mbp20*, and *mc ful2 mbp20*. The red arrowheads indicate flowers in the right two panels. White bar: 2 cm. **(B)** A developmental series of sympodial meristems of each genotype from the FM stage to an inflorescence. F: flower, FM: floral meristem, IM: inflorescence meristem, IML: IM-like, VM: vegetative meristem, L: leaf. Colored dots reflect sequential meristem initiation (red, yellow, orange, green, purple, blue), black dots highlight VMs. White bar: 200 μm.

DISCUSSION

The role of *AP1/FUL*-like genes in the regulation of tomato flowering time

In this work, we show that both *mc* single and *ful2/mbp20* double mutants show delayed flowering in the primary shoot that is enhanced in the triple mutant. A plausible explanation is that they function redundantly with a similar contribution from each gene, as the additivity of *FUL2* and *MBP20* was observed previously (Jiang et al., 2021). *FUL2/MBP20* are expressed higher than *MC* in the VM and thus may play an earlier role in the transition to flowering, but the higher expression of *MC* in the TM may compensate for this. In the sympodial shoot, flowering of the *ful2 mbp20* double mutant is mildly delayed, while it is unaffected in the *mc* single mutant. However, because sympodial flowering is massively delayed in the *mc ful2 mbp20* triple mutant, it is clear that *MC* regulates sympodial flowering time as well, although the lack of a single *mc* mutant phenotype suggests that this function is fully redundant with that of *FUL2/MBP20*. In contrast to the *mc* mutant, the single *ful2* and *mbp20* mutants do display a mild delayed sympodial flowering phenotype (Jiang et al., 2021), these genes probably contribute more to this trait, possibly because they have a higher expression level in the sympodial meristem. Our research revealed that the sympodial flowering time phenotype in the triple mutant is not due to upregulation of *SP* or *SPBG*, but probably to the upregulation of *SlCKX* genes, which will likely result in delayed cytokinin accumulation in the meristem. It is peculiar that this upregulation is very strong in the SVMs of the triple mutant, but not visible in the SVMs of the double *ful2 mbp20* mutant, while this mutant does display delayed sympodial flowering. However, as early SVMs were sampled here, in which *FUL2* and *MBP20* were relatively weakly expressed, the absence of significant *SlCKXs* upregulation may be stage related, and only become clear in later SVM or even transition stages. Future research will have to address this point, as well as further unveil the link between *MC*, *FUL2* and *MBP20* in the regulation of CK homeostasis for the regulation of sympodial shoot flowering. Together with the fact that *MC* and *FUL2/MBP20* display a similar protein-protein interaction pattern, our results suggest that all three proteins act in the same pathway, both to induce primary- and sympodial shoot flowering.

In *Arabidopsis*, *FUL* is a target of the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 15 proteins, of which the mRNA is degraded by miR156 in young plants, but accumulating in the SAM when the plant ages (Wang et al., 2009; Hyun et al., 2016). As such, *FUL* induces the transition to the reproductive phase in the age pathway together with *SOC1* in the absence of external stimulators (Wang et al., 2009; Hyun et al., 2016). Additionally, *SPLs* that are expressed later during meristem

transition, such as *SPL9* and *SPL3*, regulate *LFY*, *AP1* and *FUL* to promote FM establishment. It has been shown that LFY degrades gibberellin, so that DELLA proteins are recruited by SPL9 to induce *AP1* expression (Yamaguchi et al., 2009; Yamaguchi et al., 2014). Thus, it appears that *FUL* can be induced by SPL15 already in the vegetative meristem, while both *AP1* and *FUL* can be activated by SPLs right after the transition of the SAM to specify the FM. In addition, both *FUL* and *AP1* are targets of the FLOWERING LOCUS T (FT)-FLOWERING LOCUS D (FD) protein complex (Teper-Bamnolker and Samach, 2005; Wang et al., 2009; Jung et al., 2016), which integrates various external flowering signals such as photoperiod and temperature, and activates the key FM identity genes (Wigge et al., 2005; Ahn et al., 2006). Because *FUL2*, *MBP20* and *MC* together contribute to the regulation of flowering time, they may be all three regulated by tomato *SPL* homologs and/or *FT* homologs. Indeed, it has been unraveled that miR156-targeted *SPLs* promote flowering by activating *SFT* expression in the leaves and by activating *MC* at the SAM in tomato (Silva et al., 2019). Our data indicate that *FUL2/MBP20* are higher expressed in the SAM than *MC*, and it would be interesting to investigate whether they are also regulated in the age-pathway to induce flowering in tomato. Both the *mc* mutant flowering time phenotype and the data of Silva et al. (2019) suggest that MC is activated earlier in tomato than its ortholog *AP1* in Arabidopsis. The *FT* tomato ortholog *SFT* also encodes a mobile florigenic protein to trigger flowering, and thus it is likely that *SlFUL* and *MC* also act downstream of *SFT* in tomato, but this also requires further studies. In conclusion, while in Arabidopsis only *FUL* appears to play a role in flowering time regulation, while *AP1* is subsequently essential to specify the floral meristem, their roles in the induction of flowering seem less separated in tomato, where *MC* also plays a role in the timing of primary- and sympodial shoot flowering.

Regulation of FM and IM identity by *AP1/FUL*-like genes in tomato

The development of the WT, unbranched inflorescence of tomato requires the timely expression of FM specification genes and the subsequent onset of IM-identity genes in the newly formed flanking meristem. The key genes that specify FM identity in *Arabidopsis* are *LFY*, *UFO*, *AP1*, and to a lesser extent also *FUL* (Kaufmann et al., 2005). In tomato mutants of the orthologs of *LFY* and *UFO*, *FA* and *AN*, the FM cannot be established to form flowers, but flanking meristems massively proliferate, having IM fate in the *an* mutant, but VM fate in the *fa* mutant. This suggests that both *FA* and *AN* are required for the establishment of FM identity in tomato, and that *FA* is essential for determining IM identity as well. In contrast to the situation in Arabidopsis, the tomato IM and FM are not physically separated by a feedback loop

between *TFL1* and *AP1*, but temporally separated. The first IM is formed on the flank of the first FM, where the gradual onset of *AN* expression guides the transition to FM (Lippman et al., 2008). Earlier studies have revealed that *MC* also has a role in maintaining IM identity, as loss of *MC* leads to inflorescences that revert to vegetative growth after a few flowers. We also observed variable inflorescence vegetative reversion in *slful* mutants, which was to some extent due to environmental conditions such as high temperature (Tooke et al., 2005), and *FUL2/MBP20* mutations severely enhanced the *mc* phenotype, revealing a role for *FUL2/MBP20* in specifying the IM as well, albeit less prominent than that of *MC*. This indicates that MC, FUL2 and MBP20 are already important for IM identity before *AN* becomes expressed (and FM fate is adopted). Notably, *MC* has a prominent role later during FM development, which is not shared by *FUL2/MBP20*. In the *mc* mutant, the sepals are converted into leave-like organs, indicating that *MC* additionally functions in the specification of the first whorl of floral organs. Thus, while the vegetative reversion phenotype in the *mc ful2 mbp20* mutant can be explained by a loss of IM identity, MC also plays a later role in FM development, which does not appear to be shared by FUL2/MBP20, reminiscent of the situation in Arabidopsis. A detailed analysis of the expression patterns of *FUL2*, *MBP20* and *MC* in the reproductive meristems will shed more light on their separate and overlapping functions.

Like the *mc ful2 mbp20* triple mutant, *mc j* double mutant inflorescences reverted to vegetative growth after the formation of one flower, which is much earlier than the reversion in *mc* and *j* single mutants (Yuste-Lisbona et al., 2016b), suggesting that *MC* synergistically interacts with *J* to control IM identity (Thouet et al., 2012a; Yuste-Lisbona et al., 2016b). Because MADS-domain proteins often act in higher order complexes with other MADS-domain proteins, and both *MC* and *J* are expressed in the IM, their protein products are probably members of the same transcription factor complex. MC cannot dimerize with the SlFULs, but the SlFULs are able to interact with J, indicating that MC and FUL2/MBP20 may be interchangeable in an IM-specifying MADS-domain complex that contains J and probably other MADS-domain proteins, such as J2 and EJ2 from the SEPALLATA-clade. Interestingly, the meristem developmental dynamics show that the FM is initially specified a few times in the *mc* mutant before reversion, but only once in the *mc ful2 mbp20* mutant, although the flanking meristems of triple mutants arise like those of *ful2 mbp20*. Even though the newly forming meristems of all genotypes have the same physical dimension, similar in shape to doming TMs, their fate differs from IM (in *ful2 mbp20*) to VM/SYM (in *mc ful2 mbp20*). Because their appearance is the same, it is currently unknown at which stage the molecular changes occur that determine the fate of the

newly formed flanking meristem, and more research is required to identify the downstream factors that regulate the specification of IM and FM.

Conservation and diversification of *AP1/FUL*-like genes in plant reproductive development

In angiosperm species, only the core eudicots possess *euAP1* and *euFUL* gene clades, while non-core eudicot species only have sequences similar to *euFUL* genes (*FUL*-like), as the euAP1 proteins arose from a frame shift that occurred after the split of the eudicots, and form a derived clade within the *AP1/FUL* family (Litt and Irish, 2003). The *euAP1* genes have been implicated in the specification of perianth identity, whereas *euFUL* genes mainly function in the reproductive phase transition and fruit development (Mandel et al., 1992; Immink et al., 1999; Benlloch et al., 2006; Bemer et al., 2012; Berbel et al., 2012; Wang et al., 2019a). Because the *euAP1* and *euFUL* clades split at the base of the core eudicots, the functions of the *AP1*- and *FUL*-like genes may have diverged in the different eudicot lineages. Indeed, while both genes have largely specialized in *Arabidopsis*, with a prominent role for *AP1* in FM specification and for *FUL* in fruit development, we show here that tomato *AP1/FUL*-like genes have more overlapping functions during floral development. Research in other species has revealed that there is more often distinct overlap between the functions of *AP1/FUL*-like genes, such as in Petunia (Morel et al., 2019a), poppy (Pabón-Mora et al., 2012), and Medicago (Cheng et al., 2018). In monocot species, two clades of grass *AP1/FUL* genes, *FUL1* and *FUL2*, are present and have conserved functions in the transition to flowering and the specification of spikelet meristem identity. A prominent example of the overlapping functions in grasses are the *FUL* wheat orthologs *FUL1* (well known as *VRN1*), *FUL2*, and *FUL3,* which play redundant/overlapping roles in the regulation of flowering time, spikelet and spike development (Li et al., 2019b). However, *VRN1* is one of the central genes in the vernalization pathway to induce flowering, where *FUL2* and *FUL3* only retain some residual functionality in this process (Yan et al., 2003; Li et al., 2019b). To date, more and more *AP1/FUL*-like genes are characterized in diverse species, which will extend the understanding of their functions and may facilitate plant improvement.

MATERIALS AND METHODS

Plant materials and growing conditions

Seeds of Tomato cv. Moneyberg were germinated either on ½ MS for tissue culture transformation or on moistened filter paper for genotyping. Tissue culture

transformation was conducted in a growth chamber with 16 h light and 8 h dark at 25 °C (see protocol below). Seedlings from tissue culture and seed germination were transplanted in rockwool and cultivated in a 21 °C growth chamber (16h light/8h dark) for several weeks. Finally, plants were transferred to the greenhouse under natural light supplemented with artificial sodium lights.

Yeast two-hybrid (Y2H)

Protein-protein interaction assays in yeast were performed using the GAL4 System using Gateway vectors as described (De Folter and Immink, 2011). The coding sequences for bait proteins and prey proteins were cloned into the pDEST32 and pDEST22 vectors, respectively, and the vectors were transformed into the PJ69-4A and PJ69-4α yeast strains. The interaction screen was performed using -LWH dropout medium, supplemented with 3 mM 3-amino-1,2,4-triazole (3-AT) or -LWA dropout medium. Plates were incubated for 5 days at RT. All constructs, except for MC-AD/BD were previously generated (Jiang et al., 2021). Primer sequences used for cloning are listed in Supplemental Table S1.

CRISPR construct generation and stable tomato transformation

The construct to create mutations in the *MC* gene using CRISPR/Cas9 was generated using GoldenGate cloning and the MoClo toolkit according to (Weber et al., 2011). In brief, the online program http://www.rgenome.net/cas-designer/ (Park et al., 2015) was used for sgRNA designing. Two sgRNAs were fused to the synthetic U6 promoter as U6p::sgRNA and ligated in Level 1 constructs pICH47761 and pICH47772. The level 1 constructs pICH47732-NOSpro::NPTII::OCST, pICH47742-35S::Cas9::NOST, pICH47751-35S::GFP::ter35S, pICH47761-sgRNA1, pICH47772-sgRNA2, and the linker pICH41800 were cut/ligated into the level 2 vector pICSL4723 as described (Jiang et al., 2021). The level 2 construct was transformed into *Agrobacterium tumefaciens* C58C1 and further transformed into Moneyberg and the *ful2 mbp20* mutant (previously generated, (Jiang et al., 2021)) through *Agrobacterium tumefaciens*-mediated transformation (Van Roekel et al., 1993). Homozygous T1 transgenic plants were used for phenotypic and molecular characterization. All primers are listed in Supplemental Supplemental Table S1.

Meristem imaging

Young plants were used to dissect shoot apices using a forceps, and older leaf primordia were removed to expose the meristems under the stereomicroscope

(Stemi 508, Zeiss) with a coupled camera (AxioCam IC, Zeiss Germany). Live meristems were imaged immediately after dissection.

qPCR analysis

For qRT-PCR analysis of *SP* expression, a batch of plants was grown as a biological replicate, and the first sympodial VMs of WT, *ful2/mbp20*, and *mc/ful2/mbp20* plants were collected. At least 30 meristems were harvested for a sample by a stereomicroscope (Stemi 508, Zeiss). Three batches of plants of each genotype were grown in the greenhouse consecutively for triplicate sampling. During meristem sampling, an acetone fixation technique was used to stabilize the RNA (Park et al., 2012), and RNA was extracted using the PicoPure RNA Extraction kit (Arcturus). After DNase treatment with Ambion Turbo DNase (AM1907), cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad). Real-time RT-PCR was performed with the iQ SYBR Green Supermix from Bio-Rad with a standard 2-step program of 40 cycles, annealing at 60℃. All primer sequences are listed in Supplemental Table S1.

Accession numbers

FUL1, Solyc06g069430; *FUL2*, Solyc03g114830; *MBP10*, Solyc02g065730; *MBP20*, Solyc02g089210; *MC*: Solyc05g056620; *SP*: Solyc06g074350.

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AUTHOR CONTRIBUTIONS

M.B. conceived the project; M.B. and X.J. designed the experiments; X.J. performed the tomato CRISPR/cas9 mutagenesis, meristem stereomicroscope examination and imaging, and qPCRs; S.C. performed the yeast two-hybrid analysis. X.J. analyzed the data and prepared the figures; G.C.A., M.B. and X.J. wrote the article.

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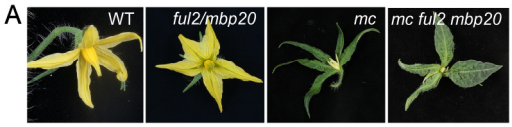
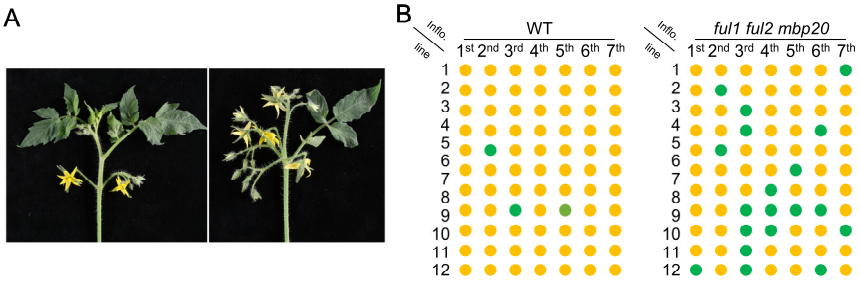
SUPPLEMENTAL DATA



Supplementary Figure 1. Flower phenotype of WT and *ap1/ful*-like mutants.



Supplementary Figure 2. Inflorescence vegetative reversion in *ful1*/*ful2/mbp20* mutants. **(A)** Representative inflorescences that reverted to shoot growth (left panel) and leaf development (right panel) in a *ful1*/*ful2/mbp20* mutant. **(B)** Frequency of inflorescence vegetative reversion in WT and *ful1*/*ful2/mbp20* mutant. Dots in yellow indicate normal inflorescences and green vegetative reverted inflorescences.

Supplemental Table S1. Primer used in this study

Experiment	Gene	Primer sequence (5'-3')
Y2H	***MC***	**F:** AAAAAAGCAGGCTTGGGAAGAGGAAAAGTTG
		R: AGAAAGCTGGGTTCATAGATGTTTATTCATGTT
CRISPR mutagenesis	*MC*-sgRNA-1 F	**F:**TGTGGTCTCAATTGTAACATTTTCAAAGAGAAGGTTT TAGAGCTAGAAATAGCAAG
	MC-sgRNA-2 F	**F:** TGTGGTCTCAATTGTTGAGTATTCTTCTG ATTCAGTTTTAGAGCTAGAAATAGCAAG
	sgRNA	**R:** TGTGGTCTCAAGCGTAATGCCAACTTTGTAC
genotyping	*SlCKX5*	**F:** TTCTTGAGGCCATTCTACACC
		R: CCATGTTTGAATGCTTGCCCA
	SlCKX6	**F:** CTCTTCACCAAAAATCACCTACTTC
		R: CCAACATCCGCGTAAAACCC
	SlCKX8	**F:** TCACTCACTTTTCTCTTCACCA
		R: CATGAACGTTACTTATTTGAGGACC
	Cas9	**F:** CTTTGGCAATATCGTGGACG
		R: CGTTCTTCTTCTCCCCAGGG
	NPT2	**F:** AGACAATCGGCTGCTCTGAT
		R: AGCCAACGCTATGTCCTGAT
RT-qPCR	*FUL2*	F: CATGAGATCTCTGTGCTTTGCG
		R: ATCCTTTCCATGCAAGAGTCAGT
	MBP20	**F:** CACATTCTCACCACCAACTTCCTAA
		R: AGTGATGAGCCTGACCCGAT
	MC	**F:** AACAGCAAGGTGAATGGCAC
		R: CCTCCTTGCTTCTGCTACTTCA
	SP	**F:** TGGACATGAATTCTTTCCTTCCTC
		R: AGGACCAAGAACATCTGGATC
	SPGB	**F:** GCACCAACCAAATGCAAGTG
		R: GCCACTTCTGATTCCAACTCG
	SlCKX5	**F:** TGGGCAAGCATTCAAACATGG
		R: AGAACAGGTCAGCATTCTGC
	SlCKX6	**F:** CTAATGCTGGAATTAGTGGTCAAAC
		R: CATATCTTTGGAGCAAGTCATTAAT
	SlCKX8	**F:** CTTCCAAAGATTTTGGAAAGATC
		R: GATAGAAAGGCCATAAGAAAATTGA
	CAC	**F:** CCTCCGTTGTGATGTAACTGG
		R: ATTGGTGGAAAGTAACATCATCG

CHAPTER 5

Enhancing tomato productivity by optimization of plant architecture

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ABSTRACT

Shoot determinacy and inflorescence complexity are two major productivity traits in tomato, which are quantitatively controlled by genes involved in the flowering pathway. An optimal balance of flowering signals will give rise to optimized plant architecture and thereby potentially increase yield. However, the available genes and allelic variation to achieve this optimum are limited. Here, we show a direct manipulation of shoot architecture by simultaneous engineering the flowering activator *MADS-BOX PROTEIN 20* (*MBP20*) and the flowering repressor *SELF-PRUNING* (*SP*). Loss of *MBP20* restores the indeterminate growth of *sp* mutants with accelerated sympodial cycling, resulting in an enhanced inflorescence complexity and compactness. The *sp mbp20* mutant plants produce more flowers and have increased fruit yield compared to wild-type plants, without any trade-off effects on fruit development. Our results provide a path to quantitatively tune the flowering signals with a direct impact on shoot architecture and productivity.

INTRODUCTION

Plant architecture is a major determinant of crop productivity. Optimization of plant architecture for higher growing density, mechanical harvesting or yield enhancement can be achieved by changes in plant stature, branching, or compactness (Doebley et al., 1997; Jin et al., 2008; Liu et al., 2010; Tian et al., 2010; Tian et al., 2019). These morphological characteristics are largely determined by the organization and activities of meristems, including the shoot apical meristem (SAM), inflorescence meristem (IM, after floral transition), floral meristem (FM) and axillary meristems (AMs). It is the meristem determinacy that shapes the shoot architecture in two basic growth habits, monopodial and sympodial. In monopodial species, such as Arabidopsis, the SAM transitions to the IM, which subsequently grows indeterminately while producing flowers to develop an inflorescence, thereby terminating vegetative growth of the shoot. The vegetative growth of sympodial plants, however, continues from axillary (sympodial) meristems when the SAM develops into a determinate inflorescence, a process that is repeated to form compound inflorescence shoots (Bell and Bryan, 2008). In sympodial plants, shoot architecture is therefore controlled by genetic mechanisms largely associated with meristem identity changes.

Plants in the Solanaceae family, particularly tomato (*Solanum lycopersicum*), are models for sympodial growth. Upon floral transition, the tomato SAM terminates into a determinate inflorescence and the shoot growth resumes from a sympodial shoot

meristem (SYM) in the axil of the youngest leaf primordium (Lippman et al., 2008a). The SYM produces three leaves and then terminates into the first flower of the next inflorescence, forming a sympodial shoot. This pattern is endlessly repeated to enable indeterminate plant growth. There is little or no variation in this indeterminate growth habit in wild tomato accessions (Peralta and Spooner, 2005), but in 1927, the *sp* mutant was discovered, in which the indeterminate shoot architecture was transformed into a determinate form (Yeager, 1927). This enabled a major agronomic shift in tomato cultivation, largely facilitating the processing of field-grown tomatoes. The responsible gene *SP* was identified as the homolog of the Arabidopsis *TERMINAL FLOWER1* (*TFL1*) gene and encodes a repressor of flowering by competing with the florigen *SINGLE FLOWER TRUSS* (*SFT*, homolog of *FT*), specifically in the SYMs (Pnueli et al., 1998; Lifschitz et al., 2006). In *sp* mutants, sympodial shoots terminate progressively faster until the cycling stops, causing compact shoots with inflorescences that develop relatively simultaneously until growth ceases (Yeager, 1927; Park et al., 2014). In contrast, loss of *SFT* arrests sympodial shoot growth and leads to highly vegetative plants with very few flowers (Molinero-Rosales et al., 2004; Krieger et al., 2010b). CRISPR/Cas9 targeting of the *SP* promoter gives rise to determinate, semi-determinate, and indeterminate plant architectures, demonstrating a high level of dosage sensitivity (Rodríguez-Leal et al., 2017). The dosage effect on *sp* determinacy can also be modified by epistatic interactions between *SP* and other florigen pathway genes. Notable is that *sft*/+ heterozygosity quantitatively compensates *sp* determinate growth, to give a semi-determinate shoot developing additional inflorescences (Jiang et al., 2013a; Park et al., 2014). Similar but weaker effects result from mutations in *SUPPRESSOR OF SP* (*SSP*, homolog of *FD*), which forms a multimeric complex with florigen to regulate the expression of floral transition genes (Park et al., 2014). The calibrating function of homologs of *SP* and *SFT* on plant architecture is highly conserved in many other crops, including soybean (Liu et al., 2010), grape (Carmona et al., 2007), sunflower (Blackman et al., 2010), cotton (Si et al., 2018), and barley (Comadran et al., 2012). Thus, it is the balance of flowering signals that regulates the consistent and perpetual sympodial cycling in tomato in a quantitative and dose-dependent way, and also regulates inflorescence shoot architecture in many other species. So far, studies to maximize yield of field-grown tomatoes by adjustment of shoot architecture have completely focused on the PEBP proteins SP and SFT. However, recent research has revealed several other genes that can influence the sympodial index and thereby plant architecture (e.g.(Jiang et al., 2021)). These form a new source of genes that may aid in gaining an optimal balance of flowering signals for maximal productivity.

An example of recently characterised tomato genes that play a role in sympodial shoot flowering time are the *APETALA1/FRUITFULL* -like (*AP1/FUL*-like) genes (see Chapter 4), which have important functions in the regulation of meristem fate in both tomato and Arabidopsis. The vegetative-to-reproductive switch in Arabidopsis is mediated by complex networks integrating environmental and endogenous signals. At the core of these networks, there is an antagonistic relationship between the key floral repressor *TFL1*, which maintains the SAM in a vegetative state, and a group of flower-inducers such as *LFY/AP1/FUL*, which are induced by the florigen FT. Loss of *TFL1* confers early flowering and results in a determinate inflorescence with a terminal flower (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1997; Ohshima et al., 1997). Mutations of *LFY*, *AP1*, *CAL*, and *FUL* allow ectopic and/or upregulated *TFL1* expression, which reverts the FM into vegetative development (Bowman et al., 1993; Liljegren et al., 1999; Ratcliffe et al., 1999; Ferrándiz et al., 2000). In tomato, loss of the *FUL*-like genes *FUL2* and *MBP20* delays flowering and promotes inflorescence complexity, and the phenotypes are dramatically enhanced by mutation of the *AP1* homolog *MACROCALYX* (*MC*), in particular in the sympodial shoots (Chapter 4). Because *SP* is in tomato only expressed in SYMs and not in the primary inflorescence meristem, and the flowering delay in *mc ful2 mbp20* triple mutants is strong in sympodial shoots, it was tempting to hypothesize that the situation in the tomato sympodial shoot would mimic that in Arabidopsis inflorescences, with mutual antagonistic functions between *SP* and the *AP1*/*FUL*-like genes. However, in Chapter 4, we show that the *SP* gene is not upregulated in *mc ful2 mbp20* SYMs, suggesting that *SP* is not repressed by *AP1*/*FUL*-like genes in tomato SYMs. Despite this, the opposite effect of the *AP1*/*FUL*-like genes on sympodial flowering time, in combination with their role in inflorescence branching, may render them perfect candidates to counteract *sp* mutations to achieve an optimal balance of flowering signals and subsequent increase in tomato yield.

In this work, we further investigated the genetic interactions between *SP* and *MBP20* and determined whether mutation of *mbp20* could lead to a more optimal balance of flowering signals in the *sp* mutant background. We focused on *MBP20* for this study, because its mutant has a mild delay in sympodial shoot flowering (on average almost 4 leaves instead of 3) and displays inflorescence branching in approximately 50% of the inflorescences, while its fruits are identical to the WT (Jiang et al., 2021). We demonstrate here that the simultaneous mutations of *SP* and *MBP20*, generated with CRISPR-Cas9, improve tomato shoot architecture and fruit yield. *sp mbp20* mutant plants exhibit largely indeterminate growth with faster flowering in the sympodial shoots, which produce more branched inflorescences and thus have improved yield.

Although there is variability in the indeterminacy phenotype, probably caused by variation in greenhouse conditions, the results indicate that the phenotype of the *sp* mutant is not dependent on *mbp20* and vice versa, suggesting that they regulate SYM activity in parallel pathways.

RESULTS

MBP20 mutation restores the indeterminate growth of *sp* mutants

To evaluate the reproductive performance of tomato plants with simultaneous mutation of *SP* and *MBP20*, we engineered *SP* knock-outs in wild-type (WT) and *mbp20* mutant background by means of the CRISPR/cas9 system (Figure 1A). The *mbp20* lines were previously generated and contained a homozygous 1 bp insertion and 13 bp deletion in the first exon (Jiang et al., 2021). The first exon of *SP* was targeted by three single-guide RNAs. The independent first-generation (T0) lines were obtained from stable transformation and analyzed by PCR and sequencing to identify the null mutations. Two presumed null mutant lines were selected, and homozygous T1 progeny plants were used for phenotypic analysis. Primary shoot flowering was unaffected in *sp* mutants compared to WT plants, but *sp mbp20* and *mbp2*0 mutant plants flowered about two leaves later than the WT in the primary shoots (Figure 1B). Generally, WT sympodial shoots flowered with three leaves (Figure 1C), while *sp* mutants were faster and stopped after producing a maximum of five inflorescences as previously reported (Pnueli et al., 1998; Park et al., 2014).

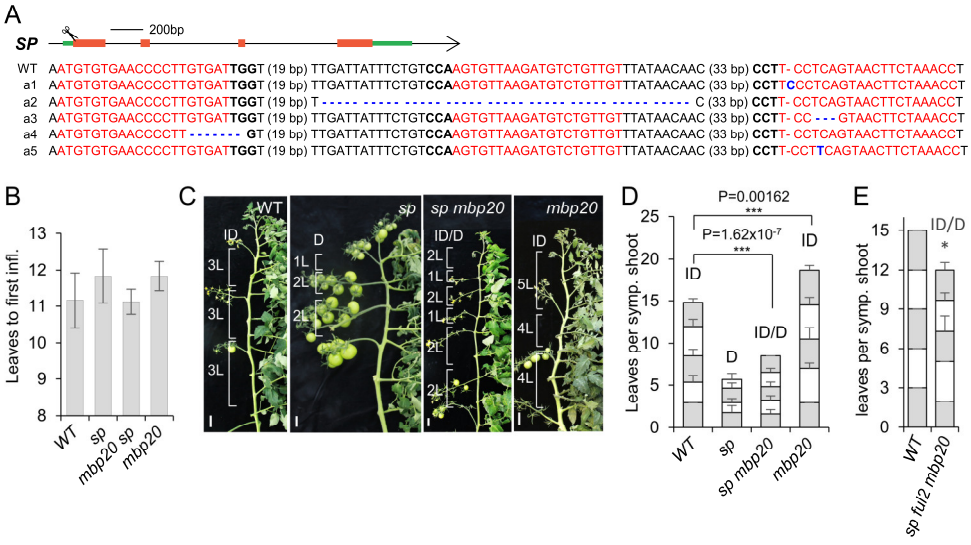

Figure 1. Mutations in *SP* and *MBP20* can be combined to create compact shoot architectures. (A) Sequences of *SP* alleles (a) obtained with CRISPR/cas9 using three sgRNAs. *sp* (a1, a2), *sp* (a3, a4)/*mbp20*, *sp* (a5)/*ful2*/*mbp20*. The *mbp20* and *mbp20/ful2* lines were previously generated (Jiang et al., 2021) **(B**) Quantification of primary shoot flowering time for wild-type (WT), *sp*, *sp mbp20*, and *mbp20* mutant plants. (**C**) Representative main shoots from all genotypes. L: leaf; D/ID: determinate, semi-determinate, and indeterminate growth. White bar: 5cm. (**D**) and **(E)** Average leaf number of leaves in sympodial shoots from all genotypes. Only the first five sympodial units were measured. The average leaf number of all sympodial shoots from each plant was used for statistical significance analysis. In B, D, and E, mean values (± SD) were compared between genotypes using one-way ANOVA followed by a post hoc LSD test, asterisks indicate the significance at P<0.05 level.

Mutation of *SP* in the *mbp20* mutant background resulted in plants with on average 1.6 leaves per sympodial shoot (in the first five analysed sympodial units), and largely restored the indeterminate growth, resulting in a higher density of inflorescences (Figure 1D). Among the nine *sp mbp20* plants analysed, three exhibited determinate growth as *sp* mutants, one terminated after producing 16 sympodial shoots (semi-determinate), and the other five plants showed indeterminate growth as did WT. To determine whether mutation of *FUL2* could further restore the sympodial leaf number and indeterminacy, we also depleted *SP* in the *ful2 mbp20* mutant background. This resulted in plants with on average 2.4 leaves in the first five sympodial units (Figure 1E). However, among the three *sp ful2 mbp20* plants, two got terminated after five and eight sympodial shoots and only one showed indeterminate growth.

Combined mutations of *MBP20* and *SP* modify plant reproductivity

In *mbp20* mutants, approximately half of the inflorescences produce two or three branches, giving rise to more flowers per inflorescence (Jiang et al., 2021). We reasoned that, if this branching would also occur in the *sp* mutant background, total flower and fruit number could be much enhanced due to the combination of more flowers per inflorescence and more compact shoot growth. To determine the yield characteristics for the different mutants, we quantified the inflorescence branching, flower number and fruit yield of each mutant. Despite an increased inflorescence complexity in *mbp20* mutants, a much smaller proportion of inflorescences branched in *sp* and *sp mbp20* mutant plants (Figure 2A), and only *sp mbp20* and *mbp20* mutant plants produced in more flowers per inflorescence than WT plants (Figure 2B). The flower production ability of these plants is consistent with their yield performance, with the highest yield for *mbp20* mutants (Figure 2 C), although this was quantified for the second and third inflorescence only and *mbp20* mutants produce less inflorescences. Unexpectedly, the yield in *sp* mutants is much lower

than that of WT for the second and third inflorescences, although they had the same flower production per inflorescence. This appears to be largely due to smaller fruits in *sp* mutants (Figure 2C). This is also reflected in the yield of the *sp mbp20* mutants, which have more flowers than the WT, but a slightly lower yield (in kg) due to smaller fruits.

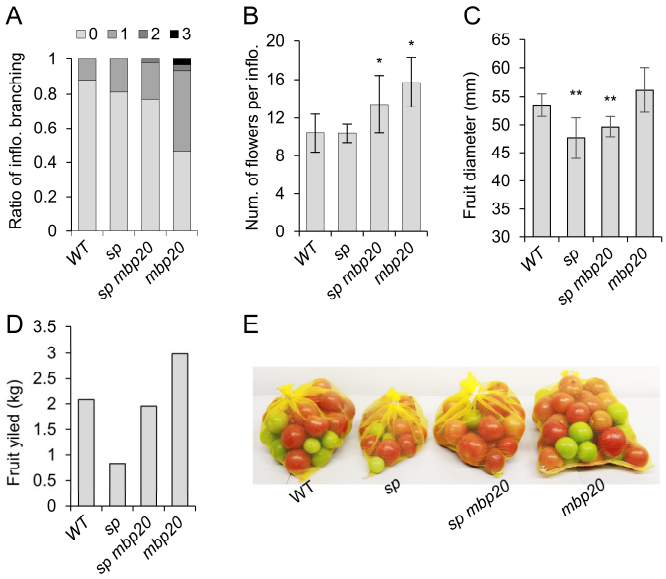

Figure 2. Yield characteristics of the different mutants. (A) Ratio of branched inflorescences in the indicated genotypes. (B) The quantification of the number of flowers per inflorescence. (**C**) fruit diameter of wild-type and mutants. **(D)** Quantification of fruit yield of the second and third inflorescences of two plants for wild-type (WT) and mutant plants. **(E)** Fruit yield of the second and third inflorescence of in total two plants. In B and C, mean values (± SD) were compared between genotypes using one-way ANOVA followed by a post hoc LSD test, asterisks indicate the significance at P<0.05 level.

DISCUSSION

Shoot architecture is a major productivity trait for tomato and depends on a dosage balance between different flowering signals. By making use of gene- and allele diversity for modification of this balance, shoot architecture can be optimized for certain growth conditions, allowing fine-tuning of sympodial cycling for yield improvement. Here, we show that loss of the tomato flowering gene *MBP20* largely rescues *sp* determinate growth, although there was variation among the double mutants, and determinate and semi-determinate growth was also observed for a few individuals. The termination of shoot growth in these individuals was likely due to

high temperatures in the greenhouse, as we observed previously in *ful2 mbp20* mutant plants grown under similar high temperatures. The intermediate shoot determinacy phenotype in the *sp mbp20* double mutant indicates that *SP* and *MBP20* are in parallel pathways regulating flowering in sympodial shoots. However, loss of *SP* only affects the flowering in sympodial shoots, but not in the primary shoot, while *MBP20* mutation delays flowering in both shoots (Figure 1A), which is consistent with the fact that *SP* is only expressed in SYM but not in the SAM, while *MBP20* is active in both meristems (Thouet et al., 2008; Jiang et al., 2021). Because *MBP20* functions in primary shoot flowering via regulation of *SlCKX*-mediated CK homeostasis, it is plausible that *MBP20* would also function via CK in the SYM, and the upregulation of the *SlCKXs* in SYMs of *mc ful2 mbp20* mutants (Chapter 4) also points in this direction. *SFT/SP* could function in parallel via the regulation of tomato flowering genes such as *FA.* The indeterminate *sp mbp20* mutant plants had faster sympodial cycling than WT, which was retarded by further depletion of the *MBP20* paralog *FUL2*, confirming the previously observed dosage effect (Figure 1D and E) (Jiang et al., 2021).

The modified shoot architecture of *sp mbp20* possessed a higher density of inflorescences with mildly increased complexity in comparison with WT plants, indicating a potential to elevate fruit yield (Figures 1D and 2A). However, the inflorescence complexity was less than that in the *mbp20* single mutant, which was unexpected. This may either be due to variation, as this trait is highly variable and only six plants were grown per phenotype or may indicate that *SP* also plays a (cryptic) role in the regulation of inflorescence structure. Inflorescence architecture is another major productivity trait also controlled by flowering genes. In Arabidopsis, the indeterminate IM fate is maintained by *TFL1* on repression of FM genes, while the FM genes in return repress *TFL1* so that a clear separation between the IM and FM is achieved (Sablowski, 2007; Liu et al., 2013; Zhu and Wagner, 2020). The loss-of-function of *TFL1* caused a dramatic conversion of the IM into an FM, producing a terminal flower (Shannon and Meeks-Wagner, 1991). In tomato, however, loss of *SP* has not been reported to influence the inflorescence phenotype, although, in our study, more branched inflorescences were observed than in the WT, the significance of this observation is doubtful due to the low number of plants.

In breeding, dramatic phenotypes are largely avoided due to possible trade-offs. For example, the *compound inflorescence* mutants produce extremely branched inflorescences with hundreds of flowers, but set fruit poorly (Lippman et al., 2008a), likely due to imbalances in source-sink relationships. However, the mild inflorescence branching phenotype in *sp mbp20* mutants resulted in a higher number of flowers and fruits, but the total weight of fruits per inflorescence was not affected

due to slightly smaller fruits. Yield, which can be defined as the total productivity of a plant, is besides the fruit production per inflorescence (Figure 2D, E) also dependent on the number of inflorescence branches. Therefore, the yield of *mbp20* mutant plants despite more fruits per inflorescence, could be mitigated due to less inflorescences per plant. In conclusion, our results suggest that different flowering genes can be used to genetically tailor antiflorigen levels for the modification of plant architecture, which may be extended to other crop species. Finding new alleles in existing germplasm or by engineering known genes would facilitate tomato breeding and de novo domestication of wild Solanaceae species and other crops.

MATERIALS AND METHODS

Plant materials and growing conditions

Seeds of Tomato cv. Moneyberg were germinated either on ½ MS for tissue culture transformation or on moistened filter paper for genotyping. Tissue culture transformation was conducted in a growth chamber with 16 h light and 8 h dark at 25 °C (see protocol below). Seedlings from tissue culture and seed germination were transplanted in rockwool and cultivated in a 21 °C growth chamber (16h light/8h dark) for several weeks. Finally, plants were transferred to the greenhouse under natural light supplemented with artificial sodium lights.

CRISPR construct generation and stable tomato transformation

The *mbp20* and *ful2 mbp20* transgenic CRISPR lines have been previously generated (Jiang et al., 2021), and their cotyledons were used, together with WT cotyledons, for transformation of an *SP* CRISPR construct. The construct was generated using GoldenGate cloning and the MoClo toolkit according to (Weber et al., 2011). Briefly, the online program http://www.rgenome.net/cas-designer/ (Park et al., 2015) was used for sgRNA design. Three sgRNAs were used to target the first exon of *SP*. Each sgRNA was fused to the synthetic U6 promoter as U6p::gRNA, and cut-ligated in Level 1 vectors. Level 1 constructs pICH47732-NOSpro::NPTII::OCST, pICH47742-35S::Cas9::NOST, pICH47751-35S::GFP::ter35S, pICH47761-gRNA1, pICH47772-gRNA2, pICH47781-gRNA3 and the linker pICH41822 were cut/ligated into the level 2 vector pICSL4723 as described. The level 2 plasmid was transformed into Agrobacterium strain C58C1. The above constructs were introduced into tomato cv Moneyberg by Agrobacterium tumefaciens-mediated transformation (Van Roekel et al., 1993). Homozygous T1 or

T2 transgenic plants were used for phenotypic and molecular characterization. All primers are listed in Supplemental Supplemental Table 1.

Genotyping and phenotyping

The PCR was conducted directly on sampled leaf tissue by using Phire Plant Direct PCR kit (Thermo Fisher) and the mutations were identified using Sanger Sequencing of the PCR fragment. Phenotypic analysis of yield component traits was performed for plants of all genotypes grown in the greenhouse. Inflorescence branching was assessed by counting the branching points in at least five inflorescences per plant. The average sympodial leaf index was determined by quantifying the number of leaves in the first five sympodial units. The number of flowers were quantified from the first four inflorescences. For fruit diameter and fruit yield, two plants per genotype were used for phenotyping. Eight fully ripe fruits were collected for size measurement. The fruit yield was the sum of the weight of red fruits and green fruits pooled from two inflorescences of each plant, and the measurements were carried out after most of the fruits were ripe.

Accession numbers

SP: Solyc06g074350; *FUL2*: Solyc03g114830; *MBP20*: Solyc02g089210.

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AUTHOR CONTRIBUTIONS

M.B. and X.J. conceived the project and designed the experiments; X.J. performed the tomato CRISPR/cas9 mutagenesis and phenotyping; X.J. analyzed the data and prepared the figures; G.C.A., M.B. and X.J. wrote the article.

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Supplemental Table 1. Primers used in this study.

Experiment	Gene	Primer sequence (5'-3')
CRISPR mutagenesis	*SP*-sgRNA-1 F	**F:**TGTGGTCTCAATTGATGTGTGAACCCCTTGTGATGTTTTAGAGCTAGAAATAGCAAG
	SP-sgRNA-2 F	**F:**TGTGGTCTCAATTGACAACAGACATCTTAACACTGTTTTAGAGCTAGAAATAGCAAG
	SP-sgRNA-3 F	**F:**TGTGGTCTCAATTGGTTTAGAAGTTACTGAGGAGTTTTAGAGCTAGAAATAGCAAG
	sgRNA	**R:** TGTGGTCTCAAGCGTAATGCCAACTTTGTAC
genotyping	*SP*	**F:** TTGTAGCACGAGAAGAGAATAACCT
		R: GTGACATAAATTAGGACAGACAGACG
	Cas9	**F:** CTTTGGCAATATCGTGGACG
		R: CGTTCTTCTTCTCCCCAGGG
	NPT2	**F:** AGACAATCGGCTGCTCTGAT
		R: AGCCAACGCTATGTCCTGAT

CHAPTER 6

General discussion

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Flowering networks in tomato

The timing of flowering is crucial for the reproductive success of plants. In tomato, the phase transition from vegetative to reproductive growth profoundly affects both the onset of inflorescence and flower formation and shoot architecture, which are regulated by overlapping gene regulatory networks. These flowering signals are generally environmental and endogenous stimuli, such as day-length, cold exposure, ambient temperature, plant age, and phytohormones, which are perceived by multiple regulatory pathways and subsequently integrated by a few key regulators (Fornara et al., 2010; Andrés and Coupland, 2012). These pathways have been largely unravelled for the model species Arabidopsis and show different levels of conservation and divergence in other plants, as flowering is species-specific. In tomato, they are partially conserved, because reduction of sensitivity to environmental effects has been one of the selection criteria for tomato domestication and breeding. For instance, domesticated tomato flowering is independent of day length as a result of allelic variations in two *FLOWERING LOCUS T (FT)* paralogs, *SELF-PRUNING 5G (SP5G)* and *FT*-like 1 (*FTL1*) for the response to long day (LD) and short day (SD), respectively (Soyk et al., 2017b; Song et al., 2020). However, many tomato flowering regulators were identified as homologs of Arabidopsis flowering genes, such as *SINGLE FLOWER TRUSS* (*SFT,* homolog of *FT*) and *PROCERA* (*PRO*, homolog of *DELLA*) (Molinero-Rosales et al., 2004; Lifschitz and Eshed, 2006; Silva et al., 2019), implying important roles of endogenous signals such as plant age and phytohormones. Here, we present an update of the tomato flowering networks, linking the data presented in this thesis to the previously published data from other studies. This involves both the regulation of the transition to flowering and the subsequent control of reproductive meristem identity. Due to the sympodial nature of tomato shoot growth, both networks are repeatedly active and interacting, but for clarity, we first discuss the regulation of the initial floral transition.

Environmental cues involved in the tomato flowering time regulatory network

The genetic variation in flowering time of wild tomato species has been exploited to reduce the sensitivity to environmental cues during domestication and enable the spread of tomato beyond its region of origin. Despite the reduced sensitivity, light conditions and ambient temperature do affect the expression of conserved flowering regulators in tomato. Photoperiod response in Arabidopsis results from the cross-talk between light perception and circadian clock, which together control the expression of the florigen FT (Andrés and Coupland, 2012). In tomato, the photoperiod response is mediated by four *FT*-like genes, *SP5G*, *SP5G2*, and *SP5G3*

and *FTL1* (Cao et al., 2016; Song et al., 2020). The silencing of *SP5G* resulted in early flowering under LD conditions and the silencing of *SP5G2* and *SP5G3* led to early flowering under SD conditions (Cao et al., 2016). In addition, the light perception and light quality (wavelength) are also crucial for plant flowering. Among the four tomato cryptochromes (*CRY1a*, *CRY1b*, *CRY2*, and *CRY3*), loss of *CRY1a* and *CRY2* perturbs the circadian clock and accelerates flowering time, probably mediated by down-regulation of *SP5G* (Perrotta et al., 2000; Fantini et al., 2019). The photoperiod response is probably also responsible for the integration of light quality information in flowering time regulation. Indeed, it was shown that a lower R:FR ratio delays tomato flowering via increased *SP5G* expression (Cao et al., 2018; Kim et al., 2019). Although classical studies have shown that low temperature induces early flowering during a nine-day sensitive phase after cotyledon expansion (Lewis, 1953; Calvert, 1957), how the ambient temperature affect tomato flowering is largely unknown. However, we observed a large variation in the number of days to flowering in our greenhouse experiments, in particular in the *mc ful2 mbp20* mutants. Because the temperature was highly variable in our greenhouse, this suggest that the influence of the temperature/light pathway is considerable, especially when the age pathway (putatively regulated via *AP1/FUL*-like genes) is absent. Thus, although the environmental pathways are of minor importance for domesticated tomatoes compared to the endogenous pathways, they are a factor that should be considered when endogenous factors are modified in breeding, for example to enhance yield.

Age-pathway

The miR156-mediated age pathway controls the competence to flower in diverse species. In young plants, a high level of miR156 prevents precocious flowering while a reduced level allows the rise of *SQUAMOSA PROMOTER BINDING PROTEIN*-LIKE (*SPL*) levels in the shoot apical meristem (SAM) to activate flower-promoting MADS-box genes, particularly *FRUITFULL* (*FUL*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)* (Wang et al., 2009; Hyun et al., 2016). Although tomato flowering is considered age-regulated, the underlying components are not well-identified yet. It has been shown that miR156 overexpression in tomato leads to late flowering and that miR156-targeted tomato *SPL* homologs (*SlSPLs*) promote flowering by activating *SFT* expression in the leaves and by activating *MACROCALYX (MC)* at the SAM (Silva et al., 2019), but other (downstream) players need to be unraveled. We showed that the tomato *AP1/FUL*-like genes *MC, FUL2*, and *MADS-BOX PROTEIN 20* (*MBP20*) together contribute to the promotion of flowering in both the primary shoot and sympodial

shoots (**Chapter 4**). Loss of them, individually or in combination, resulted in more vegetative growth before floral initiation, suggesting that they are involved in the age pathway, similar to Arabidopsis. The putative protein complex partners of MC, FUL2 and MBP20, the tomato SOC1 homologs TOMATO MADS-box gene 3 (TM3) and SISTER OF TM3 (STM3) are at the gene level probably regulated by SlSPL and SFT as well, because loss of both caused late flowering (Alonge et al., 2020a). Thus, we propose that a complex of SlFUL/MC-TM3/STM3 has a prominent role in the age pathway downstream of SlSPLs and/or SFT, and is a prominent regulator of flowering in the primary shoot. This hypothesis is further supported by our observation that in the *mc ful2 mbp20* triple mutant, flowering time is highly variable, and much more dependent on external conditions such as temperature and light than in wild-type (WT) plants where the age-pathway is still prominently active (**Chapter 4**).

Downstream of the tomato FUL-like proteins (SlFULs) and MC, tomato *cytokinin oxidases/dehydrogenases* (*SlCKXs*) are involved in the regulatory network, mediating cytokinin (CK) homeostasis. Therefore, it is very plausible that *SlFULs* are regulated directly by *SlSPLs* or indirectly by *SlSPL*-targeted *SFT*, so that the age cues are executed by CK at the cellular level (Figure 1). CK regulates cell fate and growth in the SAM and plays a role in tomato flowering. Although it has been proposed that CK is associated with flowering regulation for a long time (D'Aloia et al., 2011; Bernier, 2013), strong evidence has not yet been provided. In this work, we show that the SAM doming in tomato is accompanied by a high CK signal, in line with another observation (Steiner et al., 2020). Together with the characterization of early flowering in null *slckx* mutants (**Chapters 2 and 3**), this provides additional evidence that CK positively regulates flowering downstream of these MADS-box transcription factors. Additionally, CK has been recently proposed to promote the juvenile-to-adult phase transition via regulation of miR172 levels (Werner et al., 2021), which suggests that it could also play a role in flowering time regulation at a different level.

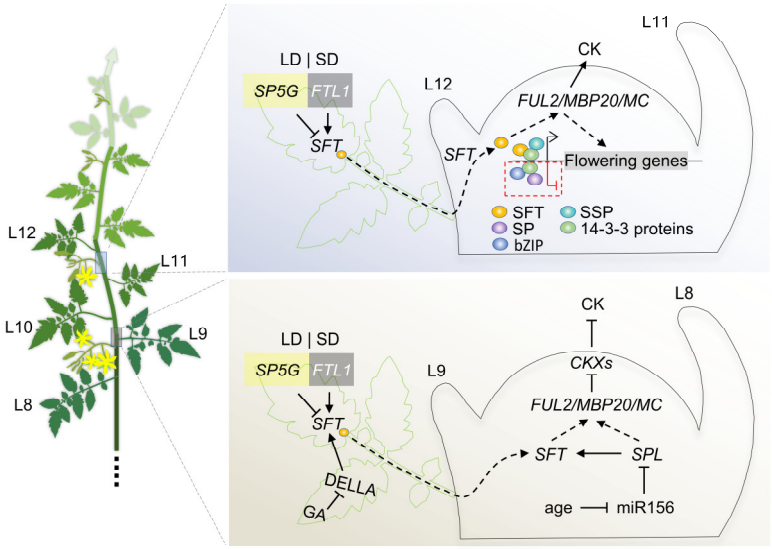

Figure 1. *AP1/FUL*-like genes involved flowering regulatory model in tomato. The model integrates results of this thesis and other work described in the text. Dashed lines indicate hypothetical interactions, while solid lines depict interactions that have been described in this thesis or other studies. L: leaf. LD: long day. SD: short day.

GA-pathway

Similar to CK, gibberellin (GA) plays a role in SAM fate and growth. The cellular behaviour of the SAM results from a heterogeneous distribution of hormones that determine cell identity and fate. Among them, the activities of GA are antagonistic to that of CK in diverse developmental processes (Greenboim-Wainberg et al., 2005; Jasinski et al., 2005; Shani et al., 2006). High levels of GA are required for the initiation and outgrowth of lateral organs, while CK in the central region of the SAM is associated with the maintenance of a stem cell reservoir. The role of GA in the regulation of the floral transition in Arabidopsis has been intensively studied. GA biosynthesis is repressed in the vegetative SAM, but upon the floral transition, GA promotes both cell division and expansion in the SAM epidermis to form the doming shape characteristic for the SAM (Kinoshita et al., 2020), a function that may be similar to that of CK in the tomato meristem (**Chapters 3 and 4**). Whether GA and CK share conserved functions in this process in both species needs to be further investigated. In this role, GA acts downstream of *SOC1*, as the *GA20ox2* is repressed by *SOC1* (Kinoshita et al., 2020). The homeostasis between CK and GA is mediated by SAM-expressed KNOXI family genes, which induce the expression of the CK biosynthesis genes, but suppress the GA biosynthesis genes (Jasinski et

al., 2005; Yanai et al., 2005; Sakamoto et al., 2006), indicating that CK-GA crosstalk may influence the floral transition.

Additionally, GA signalling, mediated via the DELLA repressor proteins, is associated with the crosstalk between various flowering pathways by upregulation of floral integrators such as *FT*, *SOC1*, and *LEAFY (LFY)* (Fornara et al., 2010). Interestingly, GA appears to have a different role in tomato flowering, since it suppresses flowering instead of inducing it. It was shown that reduced *PRO* activity or increasing GA responses delayed flowering through reduction of *SFT* expression in the leaves (Silva et al., 2019). Thus, it is possible that GA is repressing flowering in the tomato leaves, but activating doming in the SAM. More research is needed however to further elucidate the role of the GA-pathway in tomato.

Differences between the flowering regulatory networks of the primary- and sympodial shoot

The reproductive transition in sympodial shoots is much faster than that in the primary shoot, suggesting that the sympodial flowering regulatory network differs to some extent from that of the primary shoot. The main reason for this is probably that the balance between flower-promoting and flower-repressing signals, which is crucial to maintain sympodial cycling (Shalit et al., 2009), is different in both shoot types. Primary shoot flowering is highly dependent on SFT levels, which need to reach a certain threshold to induce the flowering gene regulatory cascade. However, sympodial shoot flowering occurs when SFT levels are already high in the primary shoot. To achieve vegetative growth, it may therefore be necessary to counteract the SFT florigen signals with an antiflorigen, which is also required for the monopodial Arabidopsis inflorescence. Indeed, for sympodial cycling, both *SFT* and the antiflorigen, encoded by *SELF-PRUNING* (*SP*, homolog of *TFL1*), play dominant roles in a dose-dependent manner (Shalit et al., 2009). Loss of *SFT* caused delayed flowering in the primary shoot and disruption of sympodial growth, whereas loss of *SP* only affects the flowering in the sympodial shoot, in agreement with its lack of expression in the primary shoot (Krieger et al., 2010b; Thouet et al., 2012a). The antagonism between *SP* and *SFT* is conducted by competition for bZIP transcription factors, which bind to promoters of floral identity genes (Figure 1, (Park et al., 2014; Zhu et al., 2020a). Therefore, it is likely that the difference in SP activity, in combination with the constitutive presence of SFT in sympodial meristems, explains the difference between the SAM and sympodial meristem (SYM) transitional behaviour. In addition to this, differences in expression patterns of other important flowering regulators, such as the *LFY* homolog *FALSIFLORA* (*FA*) and the *AP1/FUL*-

like genes may also contribute to the differences between primary- and sympodial shoot flowering. Our work revealed that loss of *AP1/FUL*-like genes results in late flowering in the primary shoot, but dramatically delayed flowering in sympodial shoots, suggesting a bigger role in the promotion of sympodial shoot flowering. In the *mc ful2 mbp20* mutant, this delay was progressively severe, thereby completely opposing the effect of *sp* mutation, which causes progressively earlier flowering (Yeager, 1927; Pnueli et al., 1998). How these progressive effects may be explained has to be investigated in future research. We found that the expression of several *CKX* genes is highly upregulated in SYMs of the *mc ful2 mbp20* mutant, strongly suggesting that *MC/SlFUL* also activate flowering in the SYM at least partially via regulation of CK homeostasis (Figure 1). Interestingly, combined mutations of *sp* and *slful* caused a similar dosage effect as previously found for combinations of *sft* and *sp* alleles (**Chapter 5**), indicating that the *SlFULs* are not acting up- or downstream of *SP*, but genetically independent. This would imply that the complex of *SP* with the FD-homolog *SUPPRESSOR OF SP (SSP)* is not upstream of *SlFUL*, and, given the fact that *FT* and *TFL1* compete for the same targets in Arabidopsis (Zhu et al., 2020a), that *SFT-SSP* may also not act upstream. However, much is still unclear about the regulation of (sympodial) flowering time and further investigations are required to understand all underlying and interacting mechanisms.

Regulation of reproductive meristem identity and activity

WT tomato plants develop single-branched inflorescences from sequential floral meristem (FM) maturations and inflorescence meristem (IM) initiations. Changes in this inflorescence architecture can result from differences in the rate of FM maturation and/or the fate of the newly formed flanking meristem (which adopts IM fate in the WT). The former determines the number of additional flanking meristems formed per FM, and thus the inflorescence complexity, while the latter is responsible for subsequent vegetative or reproductive growth, so that the two factors together define the nature of the inflorescence. Consequently, the inflorescence development mutants identified so far, including *macrocalyx* (*mc*), *compound inflorescences* (*s*), *anantha* (*an*) and *fa* (Allen and Sussex, 1996; Molinero-Rosales et al., 1999; Lippman et al., 2008a; Nakano et al., 2012; Yuste-Lisbona et al., 2016b), have either defect in genes that control FM identity/maturation or in IM specification, or both (Figure 2). We show that the mild delay of FM maturation in *slful* mutants occasionally allows the formation of more than one IM, resulting in mild inflorescence branching. When *MC* is additionally depleted, the delay is not enhanced, but the

flanking IMs lose their fate and adopt a vegetative fate, suggesting that *SlFUL* plays a major role in FM maturation, but a minor role in the specification of flanking meristem identity, whereas *MC* has the opposite function (**Chapter 4**). Interestingly, the loss of *SlCKXs* results in the simultaneous formation of two IMs on the FM flanks (**Chapter 3**), which may be associated with a bigger FM meristem, similar to the situation in Arabidopsis, where loss of *CKX3*/*CKX5* increased the IM size and thereby lead to more flower primordia (Bartrina et al., 2011). Notably, the Arabidopsis *MC* homolog *AP1* is suppressing CK signalling during floral organ initiation by suppressing the CK biosynthesis gene *LONELY GUY 1* (*LOG1*) and activating the *CKX3* gene, to prevent the formation of fresh meristems in the sepal axils (Han et al., 2014b). Thus, the *AP1/FUL*-like genes may be generally responsible for regulation of CK homeostasis in both the SAM/IM and FM. In turn, CK transcriptional response affects meristematic genes (Meng et al., 2017; Wang et al., 2017; Xie et al., 2018), suggesting that CK signalling, and transcription factors regulate each other reciprocally. Together, the interactions between transcription factors and hormones form the regulatory basis of meristem activity.

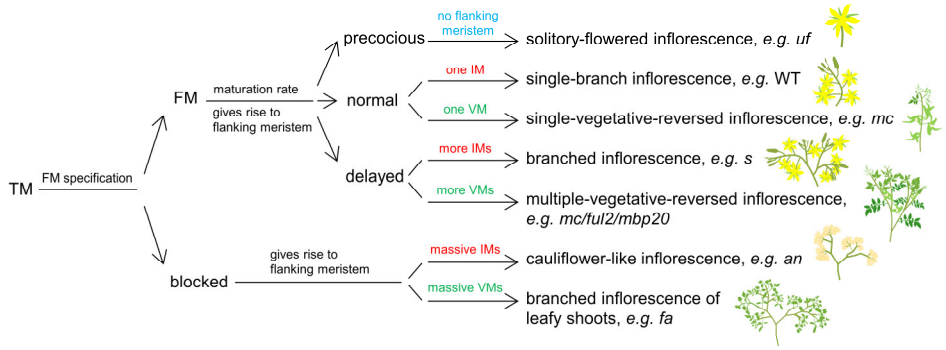

Figure 2. Specification and maturation of FM and IM shape the tomato inflorescence. The rate of FM maturation determines the number of adjacently formed flanking meristems, and the IM or VM fate that flanking meristems adopt determines reproductive or vegetative growth, respectively. TM: transition meristem, FM: floral meristem, IM: inflorescence meristem, VM: vegetative meristem. *uf*: *uniflora*, *mc*: *macrocalyx*, *s*: *compound inflorescence*, *an*: *anantha, fa*: *falsiflora*.

The FM maturation and flanking meristem initiation requires complex and well-coordinated cell division and differentiation. Therefore, unraveling where and when the flanking meristem is initiated, and when and how its vegetative or inflorescence fate is determined, will largely increase our understanding of inflorescence development. Such processes may involve intercellular communication between

cells, layers and zones of the meristem with the elaborate coordination of cell proliferation and differentiation. It will be interesting to explore how the signals move between FM and flanking meristem to program meristem activity at the cellular level. Especially, only the delayed FM maturation allows additional formations of IMs, implying that there are local signals from the FM, such as proteins or hormones, but possible also reverse signals from IM to FM, to prevent FM termination. Therefore, careful characterization of IM development, including the exact monitoring of cell division during developmental and determination of the exact timing of FM maturation, are required to gain further insight. In addition, recent advances in single-meristem genomics can be exploited further, to enable a high-resolution dissection of the temporal events (Meir et al., 2021). This will also aid in gaining more insight into the precise temporal and spatial expression patterns of key genes such as the *AP1/FUL*-like genes, which is also essential for a better understanding. To further elucidate the interaction between these key genes and hormone pathways, information about the accumulation and traffic of hormones such as auxin and CK is also required. In particular CK is of high interest, as we observed strong CK activity in both the FM and IM, and simultaneous formation of IMs in *slckx* mutants (**Chapters 2 and 3**), Intercellular CK movement is enabled by both transporters and passive distribution, of which the latter is facilitated by CKXs of different substrate preferences and expression patterns (Schmülling et al., 2003; Gajdošová et al., 2011; Zalabák et al., 2016), and studying the role of CK in meristem initiation and development will thus require a comprehensive approach.

Tuning plant architecture for crop improvement

The onset of flowering and the nature of inflorescence architecture and shoot architecture result from meristem growth dynamics. The genes controlling meristem activity have been exploited for agricultural purposes such as early flowering, more flowers, larger fruits, and shoot determinacy for mechanical harvesting. For instance, bigger fruits and higher yield can be achieved by modification of meristem size, controlled by the *CLAVATA (CLV)*-*WUCHEL* (*WUS*) circuit (Muños et al., 2011; van der Knaap et al., 2014; Xu et al., 2015). Typically, branched and compact inflorescence will lead to yield increase, and can be achieved by genetic modifications of meristem regulators, such as *S*, *JOINTLESS 2 (J2)* and *ENHANCER OF JOINTLESS 2* (*EJ2*) (Lippman et al., 2008a; Soyk et al., 2019). However, strong phenotypes obtained by deleterious mutations caused low fruit set, probably due to imbalanced source-sink relationships and reduced fertility as a result of impaired floral organ development, and thus are largely avoided during breeding. By fine-tuning the gene dosage of these meristem regulators, weak phenotypes with

high fertility can be obtained (Soyk et al., 2017a). We show that loss of *SlFUL* genes only results in weakly branched inflorescences with fully fertile flowers, although the *FUL2* mutation causes additional defects during fruit development and ripening (Bemer et al., 2012; Wang et al., 2019b). *MBP20* mutation however, had no negative effects on the fruit and is therefore an excellent target for yield improvement. Moreover, the *MBP20* mutation also restored *sp* indeterminate growth with faster sympodial cycling, to allow a combined performance of weak inflorescence branching and enhanced inflorescence compactness, which could be translated to yield enhancement (**Chapter 5**). Our work suggests that loss-of-function of a single gene also can be explored for desired reproductivity, in particular when the effect of gene mutation is relatively mild due to redundancy with homologous genes. Therefore, identification of such genes is another option in both research and breeding. Finally, although the crop germplasms harbour a rich genetic variation, finding new genes and introducing the desired alleles to different genotypic backgrounds can be challenging. Genome editing allows the introduction of genetic variation in any given background and the precise targeting of conserved networks could allow rapid crop adaptation to future cultivation practices.

Concluding remarks

In this thesis, we studied the role of *AP1/FUL*-like genes in the tomato flowering regulation and inflorescence development by characterization of their functions, redundancy and downstream targets. Even though tomato floral- and inflorescence development has been well-characterised at the morphological level, the underlying molecular mechanisms remained largely unravelled. Here, we contributed to the identification of important regulators by unveiling the role of the *SlFUL* genes in tomato flowering, and we further investigated the genetic and protein-protein interactions of the SlFUL transcription factors with other regulators to get a more comprehensive insight into the flowering regulatory network. This research increased the insight into the regulation of tomato flowering, but a lot of work still needs to be done to understand the precise activity of the key regulators in both the FM and the IM. Studying the regulatory networks at the cellular level, with a focus on the interactions between the involved transcription factors and hormone pathways would provide further insights into tomato reproductive development and increase the opportunities for of crop improvement.

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APPENDIX

Summary
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Publication

SUMMARY

Flowering time, inflorescence architecture and shoot determinacy are important features for the reproductive success of a tomato plant. These traits result from reproductive meristem activities that are regulated by networks of interacting genes. The work described in this thesis aims to extend the knowledge of the molecular-genetic mechanisms underlying flowering processes in tomato, with the focus on the on *FRUIFULL* (*FUL*)-like genes (*SlFULs*). The tomato genome contains four *SlFUL* genes (*FUL1*, *FUL2*, *MBP10*, *MBP20*), which encode for MADS domain transcription factors. It has been previously described that *FUL1* and *FUL2* have a prominent function in the regulation of fruit ripening. In this work, we studied the *SlFUL* functions in tomato flowering and inflorescence development, as well as investigated their downstream targets, functional redundancy and interactions with other proteins. We demonstrate here that *SlFULs* promote flowering through repression of tomato *cytokinin oxidases/dehydrogenases* (*SlCKXs*), thereby mediating cytokinin (CK) homeostasis. *SlFULs* and *SlCKXs* are also involved in inflorescence development, both influencing inflorescence meristem (IM) initiation, albeit probably via different mechanisms. In addition, *FUL2* and *MBP20* function redundantly with *MACROCALYX* (*MC,* homolog of *AP1*) to specify IM identity, and provide an optimal balance of flowering signals to control regular sympodial shoot flowering, in parallel with the anti-florigen *SELF-PRUNING* (*SP*, homolog of *TFL1*). Adjusting the levels of *SP* and the *SlFULs* together enables an optimization of the tomato shoot architecture for potential yield improvement.

Chapter 1 provides background on the general mechanisms of flowering and inflorescence development, with a focus on the underlying gene regulatory network. In addition to the fundamental aspects of plant reproductive development, the application of the knowledge for crop modification is also discussed.

In **Chapter 2**, we engineered loss-of-function single, double, triple and quadruple mutants of the *SlFULs* in tomato by CRISPR/cas9, and identified late flowering in most mutants in both the primary shoot and sympodial shoots due to late SAM doming. In addition, mildly enhanced inflorescence complexity because of delayed FM maturation was observed in all mutants except for *mbp10*. Transcriptome analysis of the primary shoot meristems revealed four negative regulators of CK signalling (*SlCKX5*, *SlCKX6*, *SlCKX8*, *ARR16*) that were repressed by the SlFULs. For *SlCKX6*, we could genetically confirm the role in the repression of tomato flowering. Since the *CKX* genes directly mediate CK homeostasis, we further studied the role of the *SlCKX* genes in flowering and inflorescence development in **Chapter 3**. Characterization of *slckx* null mutants revealed that *SlCKX6* alone appeared to

regulate primary shoot flowering, while all three genes were involved in the control of sympodial shoot flowering time and the regulation of inflorescence branching. Interestingly, this branching appears to be caused by the simultaneous appearance of two IMs on both flanks of the FM. We provide strong genetic evidence that CK plays a crucial role in tomato flowering and propose that this is conserved in other species.

In **Chapters 2** and **3**, we found that knock-out of both *SlFULs* and *CKXs* could lead to additional formation of IMs, but the identity of the specified IMs was not affected. However, previous studies had shown that mutation of the close *SlFUL* homolog *MC* did cause inflorescence vegetative reversion after the production of a few flowers. This sparked us to study the unique and overlapping functions of *SlFUL* and *MC* in the regulation of meristem phase change and meristem identity specification during reproductive development. In **Chapter 4**, we show that combined mutations of the *AP1*/*FUL*-like genes cause delayed FM maturation that allows additional formation of flanking meristems, which adopt a vegetative fate instead of IM fate, leading to a massively bushy inflorescence. Moreover, the late primary shoot flowering was mildly enhanced, but the sympodial shoot flowering was massively delayed in *mc ful2 mbp20* mutants. We concluded that *FUL2/MBP20* and *MC* function redundantly in both the timing of flowering and the specification of FM/IM identity, with a more prominent role for *FUL2/MBP20* in the former and for *MC* in the latter.

Based on Chapters 2 and 4, we attempted to make use of the loss of *AP1*/*FUL*-like genes to counteract mutations in the flowering repressor *SP* and achieve an optimal balance of flowering signals to optimize tomato plant architecture for yield improvement. In **Chapter 5**, we demonstrate that loss of *MBP20* restores the indeterminate growth of *sp* mutants to a large extent, and the faster sympodial cycling gave an enhanced inflorescence complexity and compactness, which resulted in more flowers and improved fruit yield. The observed intermediate phenotypes suggest that *SP* and *MBP20* act in parallel pathways in the determination of sympodial shoot flowering time.

Chapter 6 summarizes and discusses the major results of this thesis with emphasis on the regulatory mechanisms of tomato flowering and inflorescence development and provides perspectives on future research.

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