Control of inflorescence architecture in tomato by BTB/POZ transcriptional regulators

Cao Xu,1 Soon Ju Park,2 Joyce Van Eck,3 and Zachary B. Lippman1

1Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, USA; 2Division of Biological Sciences and Research Institute for Basic Science, Wonkwang University, Iksan, Jeonbuk 54538, Republic of Korea; 3The Boyce Thompson Institute, Ithaca, New York 14853, USA

Plant productivity depends on inflorescences, flower-bearing shoots that originate from the stem cell populations of shoot meristems. Inflorescence architecture determines flower production, which can vary dramatically both between and within species. In tomato plants, formation of multiflowered inflorescences depends on a precisely timed process of meristem maturation mediated by the transcription factor gene TERMINATING FLOWER (TMF), but the underlying mechanism is unknown. We show that TMF protein acts together with homologs of the Arabidopsis BLADE-ON-PETIOLE (BOP) transcriptional cofactors, defined by the conserved BTB (Broad complex, Tramtrack, and Bric-a-brac)/POZ (POX virus and zinc finger) domain. TMF and three tomato BOPs (SlBOPs) interact with themselves and each other, and TMF recruits SlBOPs to the nucleus, suggesting formation of a transcriptional complex. Like TMF, SlBOP gene expression is highest during vegetative and transitional stages of meristem maturation, and CRISPR/Cas9 elimination of SlBOP function causes pleiotropic defects, most notably simplification of inflorescences into single flowers, resembling tmf mutants. Flowering defects are enhanced in higher-order slbop tmf mutants, suggesting that SlBOPs interact with additional factors. In support of this, SlBOPs interact with TMF homologs, mutations in which cause phenotypes like slbop mutants. Our findings reveal a new flowering module defined by SlBOP–TMF family interactions that ensures a progressive meristem maturation to promote inflorescence complexity.

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Plants display remarkable diversity in when, where, and how many flowers are produced on inflorescences (Rickett 1944; Weberling 1989; Benlloch et al. 2007). Inflorescences originate from small groups of stem cells in shoot apical meristems [SAMS], which transition into inflorescence meristems [IMs] upon perceiving a combination of environmental and endogenous signals [Kobayashi and Weigel 2007, Andrés and Coupland 2012]. Depending on the species, inflorescences can be simple, like the single flowers of tulips, or highly complex, like the extraordinarily branched inflorescences of hydrangeas [Weberling 1989; Prusinkiewicz et al. 2007]. A quantitative range of complexity exists in between, and at the core of this variation are two major growth programs that influence meristem behavior in different ways following the transition to reproductive growth.

In monopodial plants such as Arabidopsis and maize, the SAM matures into a persistent reproductive state, and the IM generates flowers or inflorescence branches laterally, resulting in relatively narrow range of complexity [Hake 2008; Wang and Li 2008]. In contrast, in sympodial plants, such as tomatoes and related nightshades [Solanaceae] (Knapp et al. 2004), meristems mature into terminal flowers, and new growth continuously arises from specialized axillary [sympodial] meristems that serve as the foundation for remarkable diversity in inflorescence architecture [Park et al. 2014].

Sympodial growth is most well understood in tomato, where hundreds of multiflowered inflorescences form from the coordinated activities of sequentially maturing vegetative and reproductive sympodial meristems [Pnueli et al. 1998, Lippman et al. 2008]. In a typical plant, sympodial vegetative meristems (SYM’s) develop in the axils of...
the last leaf formed from the primary shoot meristem (PSM) and canonical axillary meristems. Each SYM develops three leaves before terminating in the first flower of a multiflowered inflorescence, and this process continues indefinitely to form compound shoots, where each shoot unit is comprised of three leaves and an inflorescence [Fnl et al. 1998]. Similarly, inflorescences are condensed compound shoots that develop from sympodial IMs (SIMs), each of which initiates one new SIM before maturing into a flower. Several iterations of SIM formation and renewal result in a linear zigzag arrangement of multiple flowers [Lippman et al. 2008]. Most tomato genotypes develop six to eight flowers on each inflorescence, but some cultivars and close wild relatives can exceed 20 flowers, and, in more distantly related wild species, inflorescences are even more floral due to branching. This diversity extends into the larger Solanaceae family, where inflorescence complexity ranges from single flowers in plants like peppers and petunias to the extremely branched inflorescences of many Solanaceous trees [Child 1979].

Our previous work on tomato inflorescence mutants and wild species has pointed to a prominent role for the process of meristem maturation in driving inflorescence diversity in sympodial plants (Park et al. 2012, 2014). Specifically, variation in the timing of maturation can modulate complexity such that a slower SIM maturation allows additional SIMs to form in each cycle, resulting in greater inflorescence complexity and vice versa. We previously described a mechanism that promotes meristem maturation in tomato, in which precise timing of activation of the homeobox gene COMPOUND INFLORESCENCE (CIF, homolog of Arabidopsis WOX9 and petunia EVG) followed by the F-box gene ANANTHA (AN; homolog of Arabidopsis UFO and petunia DOT) drives successive stages of SIM maturation to ensure that only one SIM develops in each sympodial cycle [Hepworth et al. 2006; Lippman et al. 2008; Rebocho et al. 2008; Souer et al. 2008]. When S or AN is mutated, maturation is delayed (s mutants) or never achieved (an mutants), resulting in SIM overproliferation and highly branched inflorescences [Lippman et al. 2008; Park et al. 2012]. Work in petunia revealed how AN completes the final stage of maturation [Souer et al. 2008]. Upon its late expression in the floral meristem (FM), AN protein interacts with FALSIFLORA (FA; homolog of the Arabidopsis transcription factor LFY) to form a flower specification complex, which activates floral identity genes [Koes 2008; Souer et al. 2008].

We recently discovered a new genetic pathway that represses meristem maturation to maintain a vegetative state, defined by the tomato TERMINATING FLOWER (TMF) gene. TMF encodes a member of the conserved ALOG [Arabidopsis LSH1 and Oryza G1] protein family in plants, members of which contain a DNA-binding domain and have weak transcriptional activity [Iyer and Aravind 2012; MacAlister et al. 2012, Yoshida et al. 2013]. We found that loss of TMF, one of 11 ALOG genes in tomato, causes much faster flowering and transformation of primary inflorescences into single flowers due to precocious activation of AN–FA during vegetative stages of PSM maturation [MacAlister et al. 2012]. Surprisingly, inflorescences that develop from side shoots are unaffected, suggesting that TMF functions specifically to maintain a vegetative state during PSM maturation. Notably, mutations in the closest homolog of TMF in rice simplifies panicle architecture [Yoshida et al. 2013], but loss of the closest homologs in Arabidopsis (LIGHT-SENSITIVE HYPOCOTYL3 and LIGHT-SENSITIVE HYPOCOTYL4) has no effect on inflorescences, although overexpressing these genes suppresses lateral organ differentiation [Takeda et al. 2011]. A handful of other ALOG genes in Arabidopsis and rice have reported roles in light signaling and floral organ development [Zhao et al. 2004; Yoshida et al. 2009; Cho and Zambryski 2011; Takeda et al. 2011; Sato et al. 2014]. Thus, ALOG proteins represent a new, poorly understood family of growth regulators with prominent, species-specific roles in reproductive development. In this study, we explored the mechanism by which TMF represses meristem maturation to control inflorescence architecture and flower production in sympodial plants.

Results

**Tomato BLADE-ON-PETIOLE (BOP) proteins interact with TMF**

Using TMF as bait in a yeast two-hybrid screen, we previously identified 35 interacting proteins, several of which were annotated as transcription factors/cofactors [MacAlister et al. 2012]. Among these were two homologs of the Arabidopsis BOP1 and BOP2 transcriptional coactivators, which have many reported functions in growth and development but are most recognized for their roles in leaf complexity and organ abscission [Ha 2003; Hepworth et al. 2005; Norberg et al. 2005; Ha et al. 2007; McKim et al. 2008]. BOP proteins are members of the larger NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1) protein family involved in plant defense, defined by two protein–protein interaction motifs found widely in plant and animal proteins: the BTB [Broad complex, Tramtrack, and Bric-a-brac]/POZ [POX virus and zinc finger] domain and ankyrin repeats [Fu and Dong 2013; Khan et al. 2014]. Despite numerous genetic and molecular studies on the Arabidopsis BOP genes, little is known about the factors that cooperate with BOP proteins to control diverse aspects of development [Khan et al. 2014]. For consistency, we designated the TMF-interacting tomato BOPs as SIBOP1 and SIBOP2. A homology search and phylogenetic analysis revealed SIBOP3 [Fig. 1A; Ichihashi et al. 2014] and a similar size and structure for all three SIBOP proteins [Fig. 1B]. We confirmed TMF–SIBOP1/2 interactions in yeast with full-length coding sequences and found that SIBOP3 also interacted with TMF in yeast but less strongly than SIBOP1 and SIBOP2 [Fig. 1C]. We validated these interactions using GST pull-down assays and bimolecular fluorescence complementation (BiFC) in tomato protoplasts, which further showed that all three
SlBOPs interacted with TMF in the nucleus [Fig. 1D,E; Supplemental Fig. S1].

**SlBOPs are recruited to the nucleus by TMF to enhance its transcriptional activity**

We showed previously that TMF, like other ALOG proteins, is a weak transcriptional activator (MacAlister et al. 2012), and, consistent with this, a recent comparative bioinformatics analysis of ALOG proteins revealed a DNA-binding domain with distant homology with retroposon integrase proteins (Fig. 2A; Iyer and Aravind 2012). We therefore reasoned that the role of TMF in flowering, meristem maturation, and inflorescence architecture is mediated by its transcriptional activity and that this activity might be influenced by SlBOP interactions. In *Arabidopsis*, accumulation of salicylic acid (SA) following pathogen infection induces deoligomerization of cytosolic NPR1 so that monomers can enter the nucleus and form a tetrameric “enhanceosome” with TGA family bZIP transcription factors (Boyle et al. 2009). *Arabidopsis* BOPI/2 also form homodimers and heterodimers (Jun et al. 2010), and alignment of the SlBOPs with NPR1 and BOPI/2 showed conservation of four cysteine intervals in the BTB/POZ domain that are essential for oligomerization [Supplemental Fig. S2]. As expected, the SlBOPs also homodimerized and heterodimerized (Fig. 2B). Notably, TMF also formed homodimers, which we found is critical for function, as introduction of the same point mutation from a weak allele of *tmf* (*tmf-2*) [MacAlister et al. 2012] abolished dimerization [Fig. 2A,C,D].

Given the intermolecular and intramolecular interactions between TMF and SlBOPs and considering elements of the NPR1-TGA2 mechanism as a framework, we sought evidence in support of TMF–SlBOP interactions resulting in the formation of transcriptional complexes. We first examined SlBOP subcellular localization in protoplasts. Unlike the exclusive nuclear localization of TMF, all three SlBOP-GFP fusion proteins were found in both the cytosol and the nucleus (Fig. 2E), similar to NPR1 and BOP1/2 [Kinkema et al. 2000; Hepworth et al. 2005]. We next asked whether SlBOP localization is affected by TMF interactions or vice versa by coexpressing SlBOP-GFP with TMF-mCherry in protoplasts. Significantly, all SlBOP signals shifted exclusively to the nucleus in the presence of TMF (Fig. 2F), indicating that TMF recruits SlBOPs to the nucleus. To test the functional relevance of this recruitment, we assessed transcriptional activity of SlBOP–TMF by performing a dual-luciferase assay in protoplasts (Fig. 2G). Consistent with our findings in yeast [MacAlister et al. 2012], TMF showed weak transcriptional activity (Fig. 2H). Importantly, coexpressing each SlBOP with TMF resulted in a significant modest increase in transcriptional activity, particularly for SlBOP2 (Fig. 2H). Collectively, these results suggest that interactions between TMF and the SlBOPs are the foundation for SlBOP–TMF transcriptional complexes in vivo and that their formation depends on TMF recruitment of SlBOPs to the nucleus.
The SlBOP genes are expressed dynamically during meristem maturation

The likely existence of SlBOP–TMF complexes implicates SlBOP genes in meristem maturation and thus flowering and inflorescence development. In support of this, our tomato meristem maturation atlas [Park et al. 2012] showed that all three SlBOP genes are expressed to varying degrees in major tissues, including roots, leaves, and flowers, but are most prominently expressed in meristems [Fig.
3A). Notably, like TMF, the SIBOPs are highly expressed in vegetative and transition meristem (TM) stages of PSM maturation and then decrease in the FM. Expression levels in the SYM resemble the PSM (Fig. 3A). To substantiate these dynamics and compare SIBOP and TMF expression domains, we performed mRNA in situ hybridization. At the TM stage, all three SIBOP genes were expressed in domains overlapping with TMF, at the flanks of the meristem in presumptive boundary regions, and into initiating vasculature cells (Fig. 3B–E). These similarities extended to the SYM, but, unlike TMF (F), SIBOP1 (G) and SIBOP2/3 (H) are strongly expressed in the SIM. Vascular expression is also detected (red arrowheads). (E, I) SIBOP1 and SIBOP2/3 sense probe controls. Bars, 100 µm.

**Figure 3.** SIBOP genes are expressed dynamically during meristem maturation. (A) Normalized RNA sequencing (RNA-seq) read counts for TMF, SIBOP1, SIBOP2, and SIBOP3 in major tissues, including five stages of PSM maturation [Park et al. 2012]. (EVM) Early vegetative meristem (fifth leaf initiated); (MVM) middle vegetative meristem (sixth leaf initiated); (LVM) late vegetative meristem (seventh leaf initiated); (TM) eighth (final) leaf initiated. [RPKM] reads per kilobase per million mapped reads. (B–I) In situ mRNA hybridization showing expressions of TMF, SIBOP1, and SIBOP2/3 in wild-type meristems. The top right denotes probe. TMF (B), SIBOP1 (C), and SIBOP2/3 (D) are expressed similarly at the periphery of the TM, marking lateral organ boundaries. Expressions overlap also in the SYM, but, unlike TMF (F), SIBOP1 (G) and SIBOP2/3 (H) are strongly expressed in the SIM. Vascular expression is also detected (red arrowheads). (E, I) SIBOP1 and SIBOP2/3 sense probe controls. Bars, 100 µm.

The SIBOP genes have pleiotropic roles in vegetative and reproductive development

To dissect the individual and combined developmental roles of the SIBOP genes, we created loss-of-function null mutations using CRISPR/Cas9 gene editing. Following our standard protocol [Brooks et al. 2014], we targeted the first exon of each gene with two single-guide RNAs (sgRNAs) [Fig. 4A]. Multiple independent first-generation (T0) transgenic lines screened by PCR and sequencing revealed chimeric CRISPR (CR)-slbop1, CR-slbo2, and CR-slbo3 mutants carrying insertion/deletion (indel) alleles of various sizes [Materials and Methods]. To obtain homozygous stable mutants, we sequenced T1 progeny plants and isolated two out-of-frame deletion alleles for each gene that caused premature stop codons [Fig. 4B–F]. One allele was chosen for further analyses.

Similar to Arabidopsis bop1/2 single and double mutants, all three CR-slbo mutants displayed altered leaf complexity and loss of floral organ abscission [Supplemental Fig. S3A–D]. For example, CR-slbo2 mutants showed increased leaf complexity compared with wild-type plants, primarily reflected in the formation of additional intercalary leaflets on the central rachis, perhaps due to increased meristem activity along the marginal blastozone, as in Arabidopsis bop mutants [Ha 2003; Khan et al. 2014]. In support of this, in some conditions,
Figure 4. CRISPR/Cas9-engineered mutations in SIBOP genes cause strong inflorescence defects. (A) Schematics illustrating two sgRNAs (red arrows) targeting each SIBOP gene. Black arrows represent PCR genotyping primers. (B–E) A multiflowered inflorescence typical of a wild-type plant (B) and representative inflorescences from single mutants of CR-slbop1 (C), CR-slbop2 (D), and CR-slbop3 (E). The red arrowhead in D indicates a single branching event often observed in CR-slbop2 mutants. (F) Sequences from homozygous T1 transgenic progeny plants lacking the Cas9 transgene showing CRISPR/Cas9-induced out-of-frame deletions (blue dashed lines) for CR-slbop1, CR-slbop2, and CR-slbop3, resulting in two independent null loss-of-function alleles (a1 and a2). The red font highlights sgRNA targets, and black boxes indicate protospacer-adjacent motif (PAM) sequences. (G–I) A representative shoot with two successive inflorescences from CR-slbop1/2 (G), CR-slbop2/3 (H), and CR-slbop1/3 (I) double mutants. The white star in G shows a typical CR-slbop1/2 inflorescence terminating with a leaf or reverting to a vegetative shoot. The red arrowhead in H indicates a single branching event in CR-slbop2/3 double-mutant inflorescences. (J) Sequences showing out-of-frame deletion alleles for each SIBOP gene in the CR-slbop1/2/3 triple mutant. (K) Representative CR-slbop1/2/3 triple-mutant plant showing that all multiflowered inflorescences are transformed into single- or two-flowered inflorescences (arrows). The inset shows an aborted second flower bud with bracts. (L, M) Quantification and statistical comparison of flower number per inflorescence (L) and flowering time (M) in wild-type and CR-slbop single, double, and triple mutants. Note that flower number for CR-bop1/2 plants was quantified as the flowers on each inflorescence before vegetative reversion, and flower number of CR-bop2/3 was quantified as the flowers on each inflorescence branch from branched inflorescences and also unbranched inflorescences separately. Data are means ± SD. n = 12–18 in L; n = 10–15 in M. A two-tailed, two-sample Student’s t-test was performed, and significant differences are represented by black asterisks. (**) P < 0.01; (***) P < 0.001. Red arrows indicate inflorescences. Bars: B–K, 2 cm.
we observed fusion between neighboring intercalaries and shoots forming along the rachis of CR-slbop leaves [Supplemental Fig. S3C–O]. These results suggest that the SIBOPs have overlapping roles in leaf development.

In contrast to the pronounced changes in leaf development and organ abscission, the CR-slbop mutants displayed only subtle inflorescence defects. The most obvious changes were observed in CR-slbop2, where more than half of the inflorescences underwent a single branching event and developed fewer flowers on each inflorescence compared with wild type [Fig. 4D,L]. In addition, the arrangement of flowers in both CR-slbop2 and CR-slbop3 inflorescences was frequently disrupted due to defects in pedicel orientation and increased internode length between flowers compared with wild type [Fig. 4B–E]. Reproductive defects extended to flower and fruit development; floral organs were often fused between whorls [e.g., stamens fused to carpels] in CR-slbop2 and CR-slbop3, and, consequently, fruits were misshapen and displayed scarring [Supplemental Fig. S3J–N]. Several of these reproductive defects, particularly changes in pedicel orientation and internode elongation, were reminiscent of bop mutants in Arabidopsis and barley [Xu et al. 2010; Khan et al. 2012; Jost et al. 2016]. Thus, similar to BOP genes in other species, the tomato SIBOP family functions in multiple developmental contexts [Couzigou et al. 2012; Wu et al. 2012; Ichihashi et al. 2014; Khan et al. 2014; Tavakol et al. 2015; Jost et al. 2016].

Higher-order CR-slbop mutants show enhanced leaf and inflorescence defects

Given that all three SIBOP proteins interact with TMF and that their genes share overlapping meristem expression patterns, we created higher-order mutants to determine whether the SIBOPs have redundant roles in vegetative and reproductive development. To generate all mutant combinations, we crossed T0 plants and screened the progeny of F1 plants. Notably, crossing T0 chimeric CR-slbop lines allowed us to exploit CRISPR/Cas9 to reciprocally induce mutations in the wild-type SIBOP alleles introduced in the F1 cross [Materials and Methods]. Even more, this approach allowed us to efficiently generate mutations in the closely linked SIBOP2 and SIBOP3 genes [231 kb apart on chromosome 10].

All three CR-slbop double-mutant combinations showed pleiotropic changes similar to those in the single mutants, including changes in leaf complexity and loss of organ abscission [Supplemental Fig. S3E–G]. Notably, CR-slbop1/2 and CR-slbop2/3 leaves produced even more intercalary leaflets than the single mutants. Surprisingly, CR-slbop1/3 leaves developed fewer intercalary leaflets and therefore appeared simpler. These findings suggest a more complex relationship among the SIBOPs in determining leaf complexity [Ichihashi et al. 2014].

In addition to enhanced leaf phenotypes, the CR-slbop double mutants displayed a range of modifications to inflorescence architecture. Most dramatically, CR-slbop1/2 inflorescences developed fewer flowers than wild type, reverted to a vegetative shoot on the primary inflorescence, and later formed inflorescences terminated in leaves [Fig. 4G,L]. In contrast, CR-slbop2/3 inflorescences showed no vegetative characteristics but branched more frequently than CR-slbop2 mutants alone and also developed fewer flowers [Fig. 4H,L]. CR-slbop1/3 inflorescences were more similar to wild type, but, interestingly, we found that these double mutants flowered slightly faster than wild type by one leaf [Fig. 4I,M].

The SIBOP genes act together to control inflorescence architecture and flower production

To test whether all three SIBOP genes act together to control flowering and inflorescence development, we generated triple mutants by crossing homozygous CR-slbop1/2 and CR-slbop2/3 double mutants and screening progeny from F1 plants [Materials and Methods]. Similar to CR-slbop1/3 double mutants, the triple mutants also flowered faster than the wild type [Fig. 4M]. Most strikingly, we found that all inflorescences were transformed into one or two flowers [Fig. 4J–L, Supplemental Fig. S3H]. To understand the developmental basis for this dramatic simplification of inflorescence complexity, we compared reproductive stages of meristem development from the double and triple mutants and found that CR-slbop1/2 plants initiated fewer SIMs than wild type, CR-slbop2/3 plants initiated two SIMs on the sides of the first FM, and triple mutants nearly always failed to initiate SIMs [Fig. 5]. Interestingly, loss of SIMs was frequently accompanied by the formation of small bracts. These findings, based on a complete series of CR-slbop mutants, show that all three SIBOP genes are required to initiate and perpetuate the formation and maturation of SIMs.

TMF and SIBOPs act synergistically to prevent precocious flowering and promote inflorescence complexity

Our combined molecular and genetic dissection of SIBOP function suggested a close relationship with TMF in controlling flowering, meristem maturation, and inflorescence architecture. However, there are several notable distinctions between the reproductive phenotypes of tnf and CR-slbop mutants. Specifically, tnf mutants flower much faster than CR-slbop triple mutants, after only four leaves compared with seven leaves in CR-slbop triple mutants and eight leaves in wild type [MacAlister et al. 2012]. Furthermore, only the primary inflorescence of tnf is single-flowered, and this flower often develops leaf-like sepals. In contrast, all inflorescences on CR-slbop1/2/3 triple-mutant plants develop only one to two flowers, and these flowers are morphologically much more similar to wild type than to tnf single flowers. These similarities and distinctions suggested that TMF and SIBOP functions do not completely overlap.

To explore this possibility, we performed several genetic analyses. Given that tnf phenotypes are due to precocious activation of the AN–FA complex and that driving AN expression one stage earlier than its normal activation using the promoter of the S gene closely mimics CR-slbop1/2/3 triple-mutant inflorescences [MacAlister
et al. 2012), we asked whether the CR-slbop1/2/3 reproductive phenotypes depend on AN or FA. In support of this, the FM identity genes AN and SEPALLATA3 (SEP3) were both up-regulated more than twofold in the TM stage of CR-slbop triple mutants [Fig. 6A]. This subtle change is consistent with a slightly precocious activation of AN–FA and thus adoption of floral fate during meristem maturation, explaining CR-slbop early flowering and simplified inflorescences. We tested this genetically by combining slbop with an and fa loss-of-function mutations. To reduce the complexity of this analysis, we took advantage of RNAi to simultaneously knock down the activity of all three SIBOP genes in a dominant manner (Supplemental Fig. S4). We recovered two lines showing down-regulation of all three SIBOPs with a phenotypic severity between CR-slbop1/2 double and CR-slbop1/2/3 triple mutants [Materials and Methods; Fig. 6B; Supplemental Fig. S5]. We used one of these lines to generate RNAi-slbop an and RNAi-slbop fa double mutants, and both double mutants developed branched inflorescences lacking flowers due to floral organ identity defects typical of an and fa [Lippman et al. 2008]. However, branching was not as severe as in an or fa alone, and the inflorescences of RNAi-slbop an plants often developed leaves [Supplemental Fig. S6]. Compared with the complete dependence of tmf phenotypes on AN and FA [MacAlister et al. 2012], this partial suppression suggested that the early flowering and simplified inflorescences of slbop triple mutants rely on additional factors and further points to a complex interplay between the SIBOPs and TMF in reproductive development. In support of this, we found that combining RNAi-slbop with tmf dramatically enhanced flowering and inflorescence defects. Compared with our null tmf-1 allele, which flowers after four leaves, RNAi-slbop tmf-1 mutants flowered after only two leaves and developed single-flowered inflorescences throughout the entire plant, and the sepals of these flowers were even more leaf-like [Fig. 6C–I]. We validated this synergistic interaction by combining RNAi-slbop with the weaker tmf-2 allele that is less early flowering than tmf-1 and shows weak penetrance (9%) of single-flower primary inflorescences [Supplemental Fig. S7A,D; MacAlister et al. 2012]. Significantly, RNAi-slbop tmf-2 plants also flowered after two leaves, the penetrance of single-flower primary inflorescences exceeded 90%, and all inflorescences from side shoots were transformed to one to two flowers with leaf-like sepals [Supplemental Fig. S7B–D]. Taken together, these genetic interactions demonstrate that the roles of the SIBOPs in flowering and inflorescence development do not depend exclusively on TMF and suggest that other factors are involved. In this regard, it is notable that three TMF family members (TFAMs) are expressed predominantly in meristems, with TFAM1 and TFAM2 showing expression dynamics closely resembling TMF and SIBOP1 and SIBOP2 [Supplemental Fig. S8A; Park et al. 2012]. Even more, we found that both TFAM1 and TFAM2 proteins interact with all three SIBOPs in yeast, and CRISPR/Cas9-generated null mutations in each gene result in phenotypes that match multiple defects of slbop single mutants, most significantly reduced flower production in CR-tfam1 and a high frequency of inflorescences

**Figure 5.** Meristem maturation of slbop mutants. Stereoscope images comparing PSM maturation from wild type and CR-slbop double and triple mutants. Note that the TM stage is indistinguishable, with defects arising at the FM stage when the first SIM is initiated. The wild type shows the typical zigzag reiteration of SIMs that gives rise to multiflowered inflorescences. Varying defects of SIM reiteration occur in slbop double mutants, including reduced SIM production and vegetative reversion in CR-slbop1/2, two SIMs initiated in CR-slbop2/3, and normal SIM reiteration in slbop1/3. The FM stage from CR-slbop1/2/3 shows a failure to initiate SIM reiteration, resulting in one to two flowers per inflorescence. Colored dots on stereoscope images reflect successive SIM initiation (yellow, green, blue, etc.) and FM termination (red and orange dots). The white dot indicates the first SYM in the axil of the last formed leaf on the PSM. Diagrams at the right reflect the resulting inflorescences in each genotype. The black arrow in wild type indicates continued SIM reiteration, the blue arrow and green oval in CR-slbop1/2 indicate vegetative reversion, and the dotted orange line and circle in CR-slbop1/2/3 indicate infrequent formation of a second SIM. Bars, 100 µm.
with single branching events in CR-tfam2 [Supplemental Fig. S8B–H]. In addition, CR-tfam1 mutants showed altered leaf complexity, fused floral organs, loss of floral abscission, and defects in fruit shape [Supplemental Fig. S8E, I–K]. These results suggest that, like the SIBOPs, TMF family members have independent and overlapping redundant functions and further point to the existence of multiple SIBOP–ALOG transcriptional complexes.

Discussion

Despite many studies over the last decade, surprisingly little is known about the molecular mechanisms by which the conserved BOP protein family regulates diverse aspects of plant development [Khan et al. 2014]. Indeed, only one BOP protein partner is known in Arabidopsis, a TGA family bZIP transcription factor, PERIANTHIA (PAN), which shares overlapping roles with BOP1/2 in regulating floral patterning [Hepworth et al. 2005]. Here, we showed that the tomato SIBOP family, in part through interactions with the ALOG transcriptional regulator TMF, plays a major role in flowering and inflorescence architecture by modulating meristem maturation. All BOP proteins share ankyrin repeats and the BTB/POZ domains with the NPR1 family, including four cysteine residues in the BTB/POZ domain that are important for oligomerization [Jun et al. 2010]. However, BOP proteins lack the
NPR1 domains required for SA-induced translocation and transactivation activity, raising the questions of whether there is regulated transport of cytoplasmically localized BOP proteins to the nucleus and what factors might be involved [Boyle et al. 2009; Spoel et al. 2009; Jun et al. 2010, Fu and Dong 2013]. Our findings from interaction and expression assays, including evidence that TMF recruits SIBOPs to the nucleus, strongly suggest that SIBOP–TMF complexes exist in vivo, providing the first insights into a new molecular mechanism underlying one of the many developmental roles of the BOP family [Hepworth et al. 2005; Jun et al. 2010, Khan et al. 2014].

Like BOP genes in other species, the SIBOPs seem to have unequal redundant functions in vegetative and reproductive development. For example, whereas Arabidopsis BOP1 seems to have a more prominent role in leaf development, BOP2 functions more during reproductive growth [Khan et al. 2014]. Similarly, recent work in barley has shown that two BOP homologs regulate tillering, ligule development, proximal–distal leaf patterning, and several aspects of inflorescence development, including internode length and floral organ development (Tavakol et al. 2015; Jost et al. 2016). Our data suggest that SIBOP2 is the dominant family member in tomato. However, the earlier flowering and extreme simplification of inflorescences in CR-slobop triple mutants contrasts the comparatively weak bop flowering and inflorescence phenotypes in Arabidopsis and barley [Hepworth et al. 2005; Norberg et al. 2005; Xu et al. 2010; Andres et al. 2015; Khan et al. 2015; Jost et al. 2016]. Although some inflorescence defects are shared among the three species (e.g., elongated internodes and abnormal pedicel orientation), it is striking that eliminating BOP activity in Arabidopsis results in a weak loss of IM determinacy, whereas the few-flowered inflorescences of tomato CR-slobop mutants are based on precocious meristem maturation that leads to enhanced SIM termination. This difference could reflect the evolution of distinct growth habits where the SIBOP family may have been co-opted in sympodial plants to serve as major regulators of meristem maturation, mediated in part through interactions with TMF.

However, it is telling that, unlike tmf mutants, mutations in the floral specification genes AN and FA do not completely suppress slobop flowering defects, suggesting that the SIBOPs and TMF act in partially overlapping pathways and that other factors function with SIBOP proteins to control flowering, meristem maturation, and inflorescence architecture. Indeed, we show that at least two TMF homologs interact with the SIBOPs, and CR-tfam mutants display a range of phenotypes similar to CR-slobop. These additional interactions could explain the dramatically enhanced flowering defects in RNAi-slobop tmf plants. In this scenario, when TMF is lost, SIBOPs still interact with TMF family members, and, in slobop triple mutants, TMF and family members still function on their own and/or with other transcription factors/cofactors [MacAlister et al. 2012]. Thus, the stronger phenotypes of plants lacking TMF and the SIBOPs could be due to the disruption of parallel pathways and transcriptional complexes) that regulate flowering and meristem maturation by converging on the same downstream targets. Following this logic, the enhanced early flowering in RNAi-slobop tmf plants compared with CR-slobop triple mutants and tmf might be due to even earlier activation of AN. In support of this, expressing AN in the earliest stages of meristem maturation using the TMF promoter results in plants that flower after only two leaves and develop single-flower primary inflorescences, mimicking RNAi-slobop tmf plants [Supplemental Fig. S9]. Further combined biochemical and genetic studies, including in vivo identification and functional characterization of additional partners of SIBOP and ALOG family members, along with revealing their transcriptional targets in diverse tissues, will help resolve the molecular interplay between these deeply conserved protein families in plant development.

### Materials and methods

**Plant materials and growth conditions**

The tomato (Solanum lycopersicum) cultivar M82 was used in this study. Greenhouse plants were grown under natural light with supplementation from high-pressure sodium bulbs [50 mM/m²/sec] on a 16-h light/8-h dark photoperiod. Daytime and nighttime temperatures were 26°C–28°C and 18°C–20°C, respectively.

Agrobacterium tumefaciens-mediated transformations were performed using a standard protocol [Van Eck et al. 2006], with planting and acclimation of transgenic plants performed as described [Brooks et al. 2014]. To introduce RNAi-slobop into tmf mutants, we crossed RNAi-slobop #1 to tmf-1 and tmf-2, respectively, and self-pollinated the F1 plants. We then generated tmf-1 and tmf-2 mutant plants harboring RNAi-slobop transgene by PCR from F2 progeny plants. To generate RNAi-slobop an and RNAi-slobop fa mutant plants, we crossed RNAi-slobop #1 to plants heterozygous for strong alleles of an [an-e1546] and fa [LA0854] and genotyped F1 plants to obtain an and fa heterozygous plants that also carried the RNAi-slobop transgene. The RNAi-slobop an and RNAi-slobop fa mutant plants were then obtained by genotyping F2 progeny plants for an and fa mutations and the RNAi-slobop transgene.

To express the AN gene under the promoter of TMF, we used the transactivation system [Ornitz et al. 1991; Liang et al. 2006], as done previously [MacAlister et al. 2012]. Briefly, a 3.8-kb DNA fragment immediately upstream of TMF was PCR-amplified and subcloned in front of LhG4. AN cDNA was subcloned behind a 6x tandem OP array. Both pTMF-LhG4 and OP::AN were cloned into the pART27 binary vector for transformation. LhG4 lines were crossed to the appropriate OP lines, and F1 plants (pTMF>AN) were phenotyped.

### Multiple sequence alignments and phylogenetic analysis

Full-length SIBOP1/2 gene and protein sequences were identified by BLASTN using the sequences obtained from yeast two-hybrid screening as queries against the coding sequences of ITAG 2.4 [https://blast.ncbi.nlm.nih.gov/BLAST.cgi]. SIBOP3 was obtained by BLAST searches using the sequences of SIBOP1/2 and Arabidopsis BOP1/2 as queries against the coding sequences of ITAG 2.4 [https://blast.ncbi.nlm.nih.gov/BLAST.cgi]. We identified the Oryza sativa, Selaginella moellendorfii, and Physcomitrella patens BOP family members using BLASTP through the NCBI BLAST database [https://blast.ncbi.nlm.nih.gov/BLAST.cgi].
Multiple sequence alignments were generated using SeaView 4 [Gouy et al. 2010], and a phylogenetic tree was generated from protein sequences using MAFFT [version 7.221] [Katoh and Standley 2013].

Yeast two-hybrid

Yeast two-hybrid screening was performed by Hybrigenics Services [MacAllister et al. 2012]. To confirm interactions between TMF and SlBOPs by yeast two-hybrid, the full-length coding sequence of each protein was cloned to pGBD and pGAD vectors, respectively [James et al. 1996]. The BD-TMF, BD-TFAM1, BD-TFAM2, and AD-SlBOP constructs were cotransformed into yeast strain PJ69-4A, and the yeast two-hybrid assay was carried out using the protocol as described [James et al. 1996].

Recombinant protein expression and pull-down assay

To construct the plasmids that express His-TMF and GST-SlBOP proteins, the coding sequences of TMF and SIBOP1/2/3 were subcloned into pDONR221 by BP reaction [Invitrogen] and then recombined into pET-61-DEST and pET-60-DEST [Novagen] by LR reaction [Invitrogen]. To produce recombinant proteins, each expression construct was transformed into Escherichia coli BL21 [DE3]. In brief, bacteria were cultured in LB medium at 37°C to reach 1.2 (OD600) and cooled for 30 min on ice. The cells were then continuously cultured for 15 h at 16°C after adding 0.8 mM isopropyl-b-D-thiogalactopyranoside (IPTG). Cells were then harvested and lysed by sonication. The cell lysates were centrifuged at 16,000g for 30 min, and the resulting supernatants were incubated with His Mag Sepharose Ni (GE Healthcare) for purifying His-TMF proteins and MagneGST protein purification beads [Promega] for GST alone and GST-SlBOP proteins, respectively. The proteins were then purified according to the manufacturer’s instructions.

For an in vitro GST-SlBOP pull-down assay, 0.5 mg of recombinant GST and GST-SlBOP proteins bound to MagneGST beads was blocked by 5% BSA for 2 h at 4°C followed by incubation with 0.3 mg of His-TMF for an additional 2 h at 4°C. The magnetic beads were then washed five times with ice-cold 1× PBS buffer. The bound proteins were eluted by 1× SDS-PAGE buffer with heating for 10 min at 95°C. His-TMF and GST-SlBOP proteins were detected by a monoclonal anti-His antibody (MBL Life Science) and a monoclonal anti-GST antibody (Santa Cruz Biotechnology), respectively.

Subcellular localization and BiFC assays in tomato protoplasts

To generate the transient expression constructs for subcellular localization, eCFP was fused to the C terminus of SIBOP1, SIBOP2, SIBOP3, and TMF, respectively. Fusion protein expression was driven by the cauliflower mosaic virus (CaMV) 35S promoter in transient expression vectors as described previously [Xu et al. 2012]. To produce constructs for the BiFC assays, the coding regions of TMF and SIBOPs were ligated into split CFP vectors pSCYNE and pSCYCE to generate N-CFP-TMF, C-CFP-SIBOP1, C-CFP-SIBOP2, and C-CFP-SIBOP3, respectively [Waadt et al. 2008]. The constructs for subcellular localization and BiFC assay were transfected into tomato protoplasts as described previously [Xu et al. 2015]. Cells were incubated for 12 h at 28°C before harvesting for microscopy. Imaging was performed on a Zeiss Axioplan 2 fluorescence microscope with an AxioCam camera.

Dual-luciferase assays in tomato protoplasts

For the Gal4-DNA-binding domain fusions, the TMF, SIBOP1, SIBOP2, and SIBOP3 coding sequences were cloned into the pDB vector by BamHI and KpnI restriction sites to generate the effectors [Ohta et al. 2001]. pDB empty vector served as a negative control. A Renilla luciferase [LUC] gene under the control of a CaMV 35S promoter was used as an internal control, and a firefly LUC gene under the control of four copies of the Gal4 upstream activating sequence fused to a minimal 35S promoter served as a reporter [Ohta et al. 2001]. For the transient expression in tomato protoplasts, the plasmids for effector, reporter, and internal control were cotransfected into tomato protoplasts using a standard PEG-mediated transfection protocol [Xu et al. 2015] at a plasmid ratio of 6:6:1 [effector:reporter:internal control]. After culturing for 16 h, dual-luciferase assays were performed by following the instructions of the Promega dual-luciferase reporter assay system, and the luciferase activity was measured by the GloMaxTM20-20 luminometer [Wei et al. 2009]. For data collection and statistical analyses, two independent experiments with three biological replicates per experiment were performed.

Meristem imaging, tissue collection, and quantitative RT–PCR (qRT–PCR)

Hand-dissected tomato meristems and stereomicroscope imaging of meristems were obtained according to our standard protocols [Park et al. 2012]. TM stage collection, RNA extraction, and cDNA preparation of wild type and RNAi-sibop were performed according to our previously published protocols [Park et al. 2012]. Briefly, total RNA was extracted from 20–30 meristems per biological replicate using a PicoPure RNA extraction kit [Arcturus]. Total RNA (0.5–1 μg) was treated with DNase I and used for cDNA synthesis with a SuperScript III RT kit [Invitrogen]. qRT–PCR was performed using gene-specific primers in the reaction system of iQ SYBR Green Supermix (Bio-Rad) on the CFX96 real-time system (Bio-Rad) following the manufacturer’s instructions [Supplemental Table 2]. UBIQUITIN was used as a control.

The expression patterns of SIBOP1, SIBOP2, SIBOP3, and TMF were acquired from the tomato tissue RNA sequencing [RNA-seq] database [http://tomatolab.cshl.edu/~lppmanlab2/ allexp_query.html]. RNA-seq data from different tissues [e.g., Fig. 3A] were mined from the tomato genome project transcriptome profiling data sets deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP010775 [Tomato_Genome_Consortium 2012] and from our meristem maturation atlas [Park et al. 2012].

mRNA in situ hybridization

mRNA in situ hybridization was performed using standard protocols [Jackson 1992] with slight modifications. Briefly, to generate probes of SIBOP1 and SIBOP2/3, full-length coding sequences of SIBOP1 and SIBOP2, whose coding sequences are largely identical to SIBOP3 (~86%), were amplified from cDNA using KOD Xtreme hot start DNA polymerase [Novagen], and the resulting products were ligated into StrataClone pSCA-amp/kan vector [Agilent Technologies]. Plasmids were linearized and, depending on insert orientation, T7 or T3 RNA polymerase was used for in vitro transcription [Roche]. Full-length probes were used for the hybridization. Meristem stages were as described previously [Park et al. 2012]. For fixation, meristems were hand-dissected and fixed in 4% paraformaldehyde with 0.3% Triton-X under vacuum.
CRISPR/Cas9 gene editing and genotyping and phenotyping of resulting mutants

CRISPR/Cas9 mutagenesis, plant regeneration, and greenhouse care were performed according to our standard protocols [Brooks et al. 2014]. Briefly, constructs were designed using two sgRNAs targeting the first exon of each target gene to generate various indels within coding sequences [see Supplemental Table 1 for a list of sgRNAs]. For genotyping each first-generation (T0) transgenic line, three different leaf samples were collected to capture all possible induced mutant alleles due to sectoring [chimeric], and genomic DNA was extracted using a standard protocol. Each plant was genotyped by PCR for the presence of the Cas9/sgrNA1/sgrNA2 construct with primers designed to amplify a region spanning the 3′ end of the 35S promoter and the 5′ end of Cas9. The CRISPR/Cas9 T-DNA-positive lines were further genotyped for indel mutations using a forward primer to the left of the sgrNA1 target sequence and a reverse primer to the right of the sgrNA2 target sequence [Supplemental Table 1].

PCR products from selected plants were purified for cloning into the pSC-A-amp/kan vector [Stratagene]. A minimum of eight clones per PCR product was sequenced. To generate high-order CR-slbp double mutants, crosses were conducted between each T0 single mutant, and F1 plants were self-pollinated. The F2 progeny were then screened by PCR genotyping and sequencing as described above. To generate all combinations of CR-slbp double mutants, we took advantage of T0 chimeric plants of CR-slbp single mutants in which CRISPR/Cas9 can reciprocally induce mutations in the F1 cross, and this strategy also allowed us to efficiently introduce mutations in the closely linked SIBOP2 and SIBOP3 genes. The CR-slbp double mutants were obtained by genotyping the progeny of F1 plants. To create triple mutants, crosses were made between genotyping confirmed F1 double-mutant plants for CR-slbp1/2 and CR-slbp2/3, which allowed immediate creation of homozygous or bialleic mutations in SIBOP2. The CR-slbp1/2/3 triple mutants were then obtained by screening the progeny of the F1 plants.

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References


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Cao Xu, Soon Ju Park, Joyce Van Eck, et al.

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