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Involvement of the tomato BBX16 and BBX17 microProteins in reproductive development

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ABSTRACT

BBXs are B-Box zinc finger proteins that can act as transcription factors and regulators of protein complexes. Several BBX proteins play important roles in plant development. Two *Arabidopsis thaliana* microProteins belonging to the BBX family, named miP1a and miP1b, homotypically interact with and modulate the activity of other BBX proteins, including CONSTANS, which transcriptionally activates the florigen, *FLOWERING LOCUS T*. *Arabidopsis* plants overexpressing miP1a and miP1b showed delayed flowering. In tomato, the closest homologs of miP1a and miP1b are the microProteins *Sl*BBX16 and *Sl*BBX17. This study was aimed at investigating whether the constitutive expression of *Sl*BBX16/17 in *Arabidopsis* and tomato impacted reproductive development. The heterologous expression of the two tomato microProteins in *Arabidopsis* caused a delay in the flowering transition; however, the effect was weaker than that observed when the native miP1a/b were overexpressed. In tomato, overexpression of *SlBBX17* prolonged the flowering period; this effect was accompanied by downregulation of the flowering inhibitors *Self Pruning* (*SP*) and *SP5G*. *Sl*BBX16 and *Sl*BBX17 can hetero-oligomerize with TCMP-2, a cystine-knot peptide involved in flowering pattern regulation and early fruit development in tomato. The increased expression of both microProteins also caused alterations in tomato fruit development: we observed in the case of *SlBBX17* a decrease in the number and size of ripe fruits as compared to WT plants, while for *SlBBX16*, a delay in fruit production up to the breaker stage. These effects were associated with changes in the expression of GA-responsive genes.

1. Introduction

Crop productivity is the result of the interaction between the plant genetic background, the environmental cues and the agronomic practices. In the face of climate change, it becomes increasingly important to match the phenology of crop cultivars with the environmental conditions to maintain high yields. Therefore, the availability of cultivars with different phenological requirements could be advantageous for optimising plant reproduction and productivity. In horticultural plants, flowering, fruit set and the onset of ripening are processes markedly affected by temperature and photoperiod, and regulated by internal signals such as hormones, transcription factors and adaptor molecules. Among the different regulatory proteins known to control reproductive development, members of the B-Box (BBX) protein family have emerged as important molecular players that integrate environmental cues, for instance, light and temperature, with endogenous signaling pathways (Gangappa and Botto, 2014; Yadav et al., 2020).

The BBX family represents a group of zinc (Zn)-finger proteins that are involved not only in reproductive development, but also in many other physiological processes, such as photomorphogenesis (Fan et al., 2012), anthocyanin accumulation, seed germination, carotenoid biosynthesis, and responses to biotic and abiotic stresses (Gangappa and Botto, 2014; Kiełbowicz-Matuk et al., 2014; Xu et al., 2022). The BBX family is characterized by one or two Zn-finger-containing BBX domain (s) in the N-terminal region (Gangappa and Botto, 2014). Previous studies suggested that the B-Box domain plays a crucial role in

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protein-protein interactions. Some BBX proteins may possess a CCT domain, which is associated with a role in transcriptional regulation and nuclear transport (Crocco and Botto, 2013). CCT-containing BBXs are likely transcription factors, while BBXs lacking CCT domain might have other functions linked to their protein-protein interaction capacity. *Arabidopsis* BBX proteins have been classified into five subgroups according to different combinations of the above-mentioned domains. Members of group I are characterized by the presence of B1, B2, and CCT domains, as are members of group II; however, some differences have been observed between the two groups in the B2 consensus sequences (Crocco and Botto, 2013; Gangappa and Botto, 2014). The presence of B1 and CCT, B1 and B2, and a single B1 domain characterizes members of groups III, IV, and V, respectively (Gangappa and Botto, 2014). Similarly, following structural features, BBX proteins from other species, such as tomato and rice, have been grouped into subfamilies (Huang et al., 2012; Chu et al., 2016).

A. thaliana CONSTANS (*At*CO), one of the first BBX proteins to be identified and characterized, belongs to subgroup I. *At*CO plays a crucial role in photoperiodic control of flowering time, enabling the transcriptional activation of the *FLOWERING LOCUS T* (*FT*) in response to long day (LD) conditions. Proteins homologous to *At*CO have been shown to contribute to flowering regulation in other species also with different photoperiodic requirements (Campoli et al., 2012; Yang et al., 2014; Wang et al., 2019).

Other members of the *Arabidopsis* BBX family were successively discovered to be implicated in flowering control (Li et al., 2014; Tripathi et al., 2017). Recently, two *Arabidopsis* BBX proteins of the group V, microProtein (miP) miP1a and miP1b (also referred to as *At*BBX31 and *At*BBX30, respectively), were shown to modulate *At*CO activity (Graeff et al., 2016; Rodrigues et al., 2021). Both miP1a and miP1b can interact with *At*CO and TOPLESS (TPL), leading to the formation of a trimeric complex that limits *At*CO-mediated induction of *FT* expression. Consistently, *Arabidopsis* plants overexpressing miP1a and miP1b, grown under LD conditions, showed delayed flowering (Graeff et al., 2016). In tomato, the microProteins *Sl*BBX16 and *Sl*BBX17 are the closest homologs of miP1a and miP1b, but their role in tomato reproductive development is largely elusive. The features of group V BBXs - a single B-Box domain and the lack of CCT - suggest they act as microProteins regulating larger multidomain complexes at the post-translational level (Eguen et al., 2015).

In this regard, *Sl*BBX16 interacted with the tomato cystine-knot peptide 2 (TCMP-2) (Molesini et al., 2020). TCMP-2 is specifically expressed in reproductive organs, its expression is low in pre-anthesis flower buds and gradually increases after fertilization, reaching a maximum in green and ripe fruits (Cavallini et al., 2011). Increased TCMP-2 expression in pre-anthesis flower buds has been demonstrated to lead to altered flowering pattern as well as early fruit production and a slight delay in the initiation of ripening (Molesini et al., 2018, 2020).

In tomato, which is a day-neutral species, the flowering transition is principally regulated by the balance between the activity of the florigen *Single Flower Truss* (*SFT*), which is the ortholog of *FT*, and that of anti-florigens, such as *Self Pruning* (*SP*) and *SP5G*, which maintain vegetative growth. Recently, it has been shown that *Sl*COL1, the ortholog of *At*CO, is able to bind the promoter region of *SFT* to repress its expression (Cui et al., 2022). Accordingly, RNA silencing of *SlCOL1* led to the promotion of flowering and increased fruit yield (Cui et al., 2022).

The aim of this work was to evaluate the impact of the constitutive expression of *Sl*BBX16 and *Sl*BBX17 on flowering and fruit development. We demonstrated that *SlBBX17* overexpressing (*Sl*BBX17OE) plants show a prolonged period of flowering associated with a reduced expression of the *SP* and *SP5G* flowering inhibitors. Furthermore, we observed a delay in the early phases of fruit growth in *Sl*BBX16OE plants and changes in ripening in *Sl*BBX17OE plants. The overexpression of both microProteins induced modifications in the expression pattern of genes regulating gibberellin (GA) metabolism and signaling.

2. Materials and methods

2.1. Plant materials

Solanum lycopersicum MicroTom WT seeds (ID:TOMJPF00001) were obtained from the TOMATOMA mutant archive (Saito et al., 2011). *Arabidopsis thaliana* WT (ecotype Col-0) and *At*miP1a/b double KO mutant plants were employed for flowering time assessment.

2.2. Plant genetic transformation

The tomato DNA sequences corresponding to the coding regions of *SlBBX16* (Solyc12g005750) and *SlBBX17* (Solyc07g052620) were amplified by PCR from cDNAs using the primers reported in Table S1. The DNA fragments were subcloned into the pGEM®-T Easy Vector (Promega) and checked by sequencing. The coding regions of *SlBBX16* and *SlBBX17* were then cloned into a derivative of the pBin19 vector, under the control of the *CaMV35S* promoter and the terminator sequence of the *Agrobacterium tumefaciens* nopaline synthase gene.

For the overexpression of *TCMP-2* (Solyc07g049140) in MicroTom, a sequence corresponding to the coding region was amplified using the Gateway System (Invitrogen) (Table S1). After subcloning in the pDONR221, the resulting pENTRY vector was checked by sequencing and used for recombination in the destination vector pK7WG2D.1 (Karimi et al., 2002). The recombinant vectors obtained were introduced into *Agrobacterium tumefaciens* cells (strain GV2260). The genetic transformation of MicroTom was obtained from cotyledon explants of 8-day-old seedlings. *A. tumefaciens* cells were grown at 28 °C for 24 h and used at OD_{600} of 0.1 for explant infection. After 48 h of co-cultivation, the explants were transferred to Murashige & Skoog (MS) medium containing NAA (0.01 mgL^{-1}), zeatin riboside (2 mgL^{-1}) and kanamycin (100 mgL^{-1}). The regenerated shoots were transferred to the rooting medium (half-strength MS including Nitsch vitamins, sucrose 10 gL^{-1}, agar 4 gL^{-1}, phytagel 3 gL^{-1}, pH 5.8) supplemented with kanamycin (75 mgL^{-1}). After 3–4 weeks, the rooted plants were acclimatized in the greenhouse. Monitoring the ploidy level of putative transformants according to Atarés et al. (2011), we demonstrated that with this transformation method, the vast majority (about 90%) of MicroTom transformed lines retained the diploid state. *Arabidopsis* plants were transformed using the floral dip method.

2.3. Phenotypic analysis

Tomato plants were grown in the greenhouse during springtime. For phenotypic assessment, plants of the T1 generation were grown in pots and transgenic state was confirmed by spraying with kanamycin (400 mgL^{-1}). Various flowering and fruiting parameters were recorded, and the fruit yield was evaluated at about 110 days after sowing.

Arabidopsis plants were grown in a climatic chamber at a constant temperature of 25 °C under LD conditions (16/8 h light/dark cycle, photosynthetic photon fluence rate of 150 μmol m^{-2} s^{-1}). Homozygous plants of the T3 generation were used for flowering time analysis.

2.4. Yeast two-Hybrid analysis

To examine protein-protein interactions, the Matchmaker Gold Yeast Two-Hybrid System (Clontech) was used, following the manufacturer's instructions with minor modifications. To test the interaction between TCMP-2 and *Sl*BBX17, the DNA sequence of the mature portion of the TCMP-2 protein (Solyc07g049140; from amino acid 53 to 96) was expressed as a fusion with the DNA-binding domain of GAL4 in the pGBKT7-BD vector, while the entire coding region of *SlBBX17* was cloned in frame into pGADT7-AD. For the interactions between *Sl*BBX16, *Sl*BBX17, *At*CO (*At*5g15840) and *Sl*COL1 (Solyc02g089540), the entire coding regions of the BBX genes were cloned in frame into the pGBKT7-BD vector and *At*CO and *Sl*COL1 were cloned in the pGADT7-

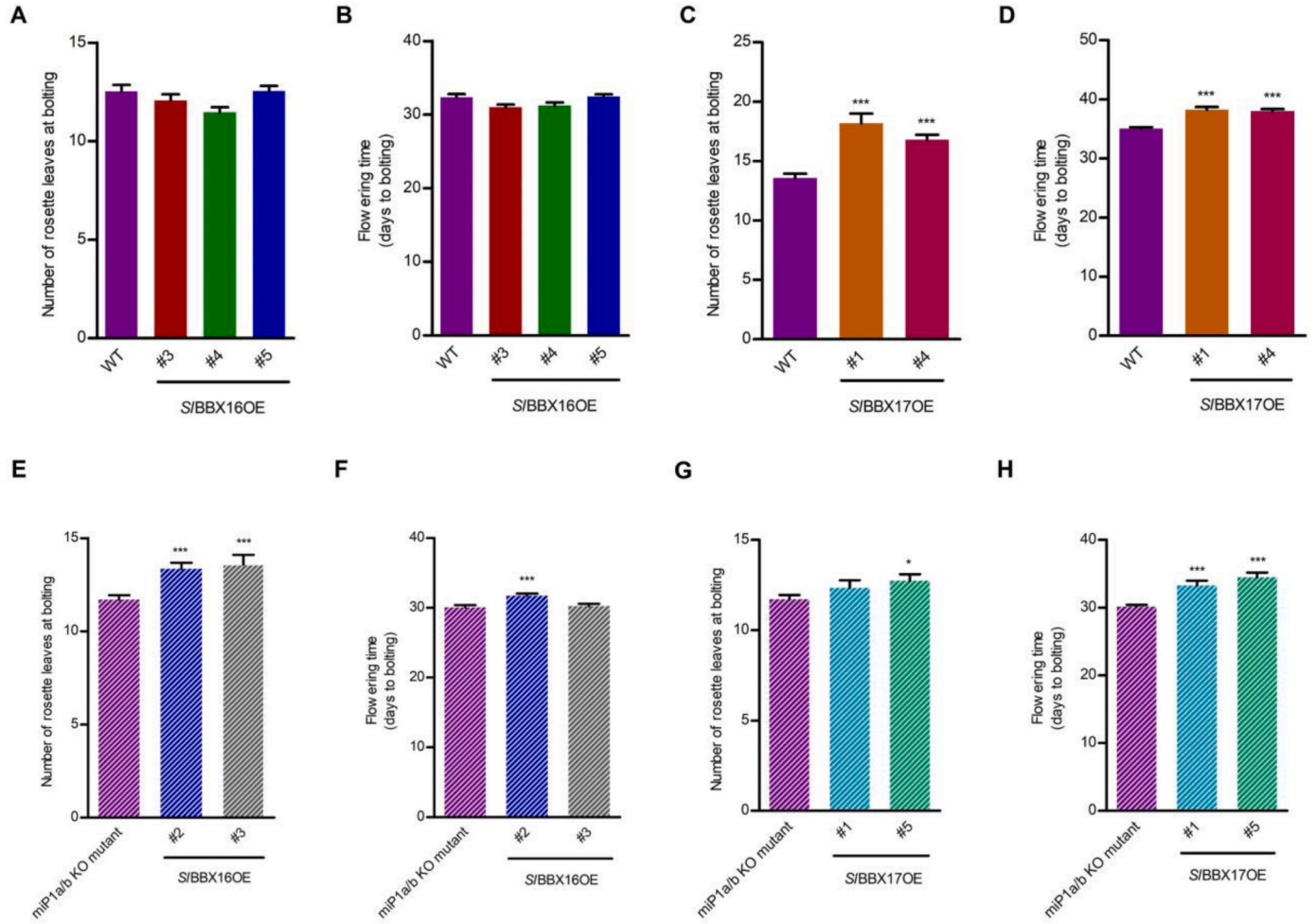

Fig. 1. Overexpression of *SlBBX16* and *SlBBX17* in *Arabidopsis* WT (Col-0) and miP1a/b KO mutant. The transition from vegetative to reproductive development was determined by counting the number of rosette leaves at the bolting stage and the number of days to bolting. (A, B) Three lines overexpressing *SlBBX16* (*Sl*BBX16OE #3, #4, and #5) and (C, D) two lines overexpressing *SlBBX17* (*Sl*BBX17OE #1 and #4) were compared with WT. Values are means ± SE (n = 19–27 for panels A and B; n = 16–26 for panels C and D). (E, F) Two *Sl*BBX16OE lines (#2 and #3) and (G, H) two *Sl*BBX17OE lines (#1 and #5) were compared with miP1a/b double KO mutant. Values are means ± SE (n = 13–26 for panels E and F; n = 18–26 for panels G and H). *P < 0.05 and ***P < 0.001 versus the respective control (Student's t-test).

AD vector. The interaction between miP1a (*At*3g21890) and *At*CO represents the positive control. For negative controls, pGBKT7 without insert (BD alone; Empty) and pGADT7 without insert (AD alone; Empty) were used. The primers employed for the genetic constructs preparation are reported in Table S1.

2.5. RT-qPCR analysis

Total RNA extraction was performed from leaves, floral organs and fruits using the "NucleoSpin RNA Plant" kit (Macherey-Nagel). After DNase I treatment, first-strand cDNA was synthesized using the ImProm-II Reverse Transcriptase (Promega). Three cDNA samples derived from three independent RNA extractions were synthesized and amplified using Luna®Universal qPCR Master Mix (New England Biolabs) on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). The data were normalized using *a*ctin (Solyc11g005330) or *SAND* (Solyc03g115810) as references for leaf and fruit tomato samples, respectively, and actin (*At*3g18780) for genes expressed in *Arabidopsis* (Table S1). Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method as previously described (Molesini et al., 2018).

2.6. GA treatment and quantification

To test the responsiveness of *SlBBX16* and *SlBBX17* to the exogenous application of GA (GA_3), tomato seedlings were grown *in vitro* for one month on half-strength MS agar medium (pH 5.9). Plants were transferred to a half-strength MS liquid solution (pH 5.9) and the next day, treated for 24 h with 5 μM GA_3. The shoots were collected for expression analysis by RT-qPCR.

GAs extractions and quantifications from tomato immature green fruits were conducted at the Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia, using Q-Exactive mass spectrometer coupled to Ultra HighPerformance Liquid Chromatography. Each biological replicate was obtained by pooling 3–5 immature green fruits collected from at least four individual plants.

Hormonal treatment of the fruits was performed according to Li and collaborators (2019). A volume of 50 μl of H_2O (control) or 0.1 mM GA_3 was injected into the pericarp of fruits (approximately 2.0 cm in diameter) near the sepals using a micro syringe. Fruits were kept on plants for 20 days before being collected.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism version

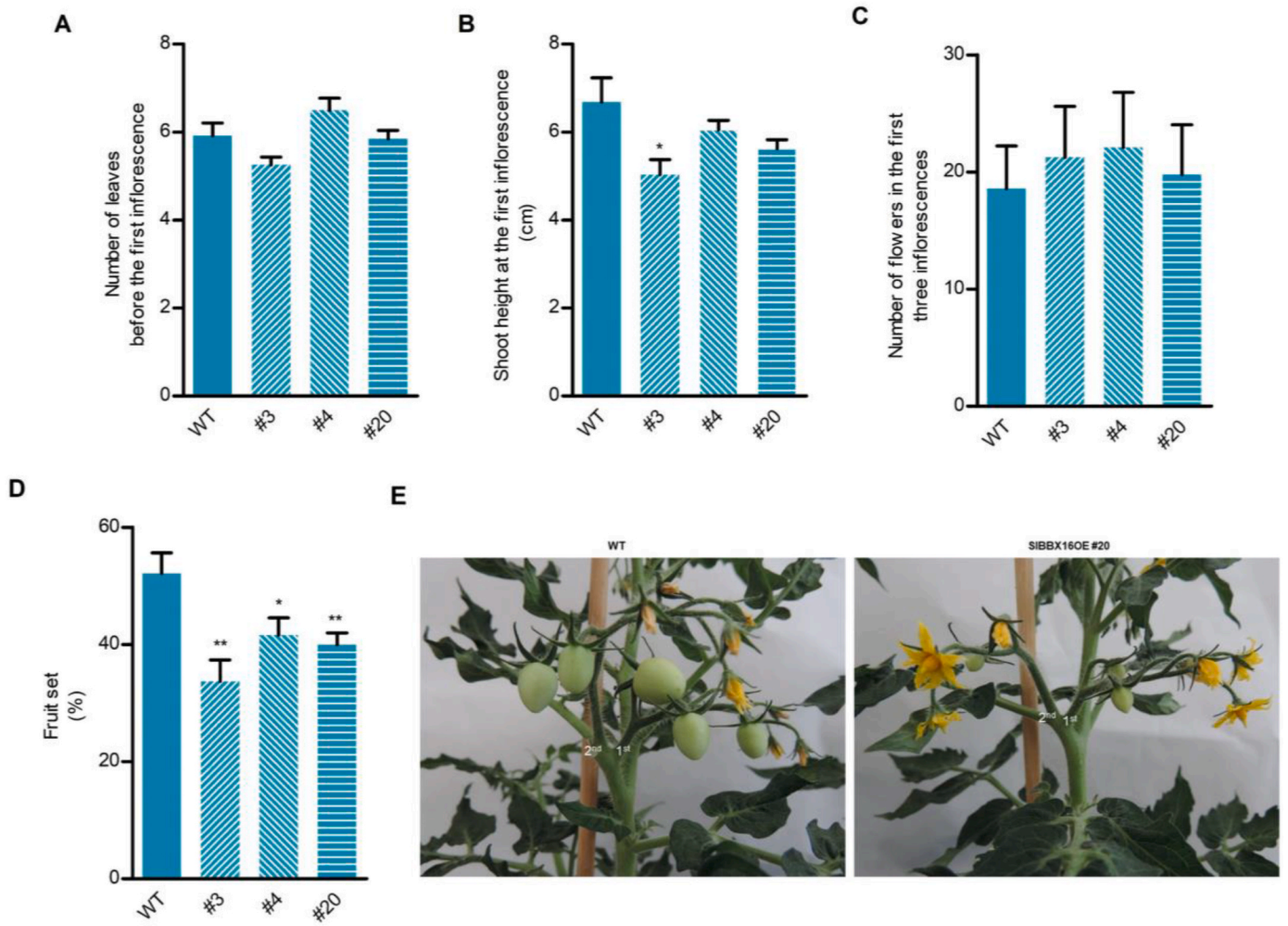

Fig. 2. Transition to flowering and fruit set parameters of *Sl*BBX16OE plants. (A) Number of leaves before the first inflorescence (B) Shoot height at the first inflorescence. (C) Number of flowers in the first three inflorescences. (D) Fruit set of the first three inflorescences calculated as the percentage of the number of fruits over the number of flowers. (E) Representative pictures of the first two inflorescences of WT and *Sl*BBX16OE #20 plants of the same age. The values reported are means ± SE (n = 10–13). Student's t-test was used for the statistical analysis (*P < 0.05; **P < 0.01).

5.0 software (GraphPad Software). Data were compared using Student's t-test.

3. Results

3.1. Ectopic overexpression of SlBBX16 and SlBBX17 influences the flowering transition in Arabidopsis

The microProteins *Sl*BBX16 and *Sl*BBX17 show high sequence similarity to miP1a and miP1b (Fig. S1A), which control flowering in *Arabidopsis* and, when overexpressed, delay flowering (Graeff et al., 2016). To test whether *Sl*BBX16 and *Sl*BBX17 can exert a similar effect, we analysed the flowering behaviour of *Arabidopsis* *Sl*BBX16OE and *Sl*BBX17OE plants compared to Col-0 WT (Fig. 1 and Figs. S1B and C). In the *Sl*BBX16OE plants, the number of rosette leaves at bolting did not differ from the control plants (Fig. 1A). Also, the days from sowing to flowering did not vary (Fig. 1B). On the other hand, both *Sl*BBX17OE lines displayed an increased number of rosette leaves at bolting and a longer time to reach flowering under LD photoperiodic conditions (Fig. 1C and D). The observed inhibitory effect of *Sl*BBX17 on flowering was less pronounced than that produced by overexpressing miP1a and miP1b in *Arabidopsis* (Graeff et al., 2016).

To examine whether *Sl*BBX16 and *Sl*BBX17 can substitute the function of miP1a/b, we expressed them also in *Arabidopsis* miP1a/b double KO mutant, which displays earlier flowering than WT (Heng et al., 2019). In both *Sl*BBX16OE lines and in the *Sl*BBX17OE #5, the number of leaves at bolting (Fig. 1E and G) increased significantly (*Sl*BBX16OE #2 13.3 ± 1.5; #3 13.6 ± 1.9; *Sl*BBX17OE #5 12.7 ± 1.6) compared to miP1a/b double KO mutant (11.7 ± 1.2) and was slightly lower than or similar to Col-0 WT (13.6 ± 0.3). The number of days to bolting was increased significantly in *Sl*BBX16OE #2 (31.7 ± 1.6) and in both *Sl*BBX17OE lines (33.2 ± 3.3 and 34.4 ± 3.1 days for #1 and #5, respectively) (Fig. 1F and H) reaching values similar to those observed in Col-0 WT (Fig. 1B and D). These results suggest that *Sl*BBX16 and *Sl*BBX17 may impact the flowering transition in *Arabidopsis* and partially rescue the function of endogenous miP1a/b. Since the flowering delay exhibited by *Sl*BBX16OE and *Sl*BBX17OE plants resembles, albeit in an attenuated form, the phenotype shown by miP1a/b-overexpressing plants (Graeff et al., 2016), we examined via Y2H whether *Sl*BBX16 and *Sl*BBX17 interact with *At*CO as already demonstrated for miP1a/b (Graeff et al., 2016). Under our experimental conditions, we did not observe a direct interaction between *Sl*BBX16 and *Sl*BBX17 and *At*CO (Fig. S2).

3.2. SlBBX16 and SlBBX17 and tomato reproductive development

We recently demonstrated that *Sl*BBX16 interacts with TCMP-2, a tomato cystine-knot metallocarboxypeptidase inhibitor (Molesini et al.,

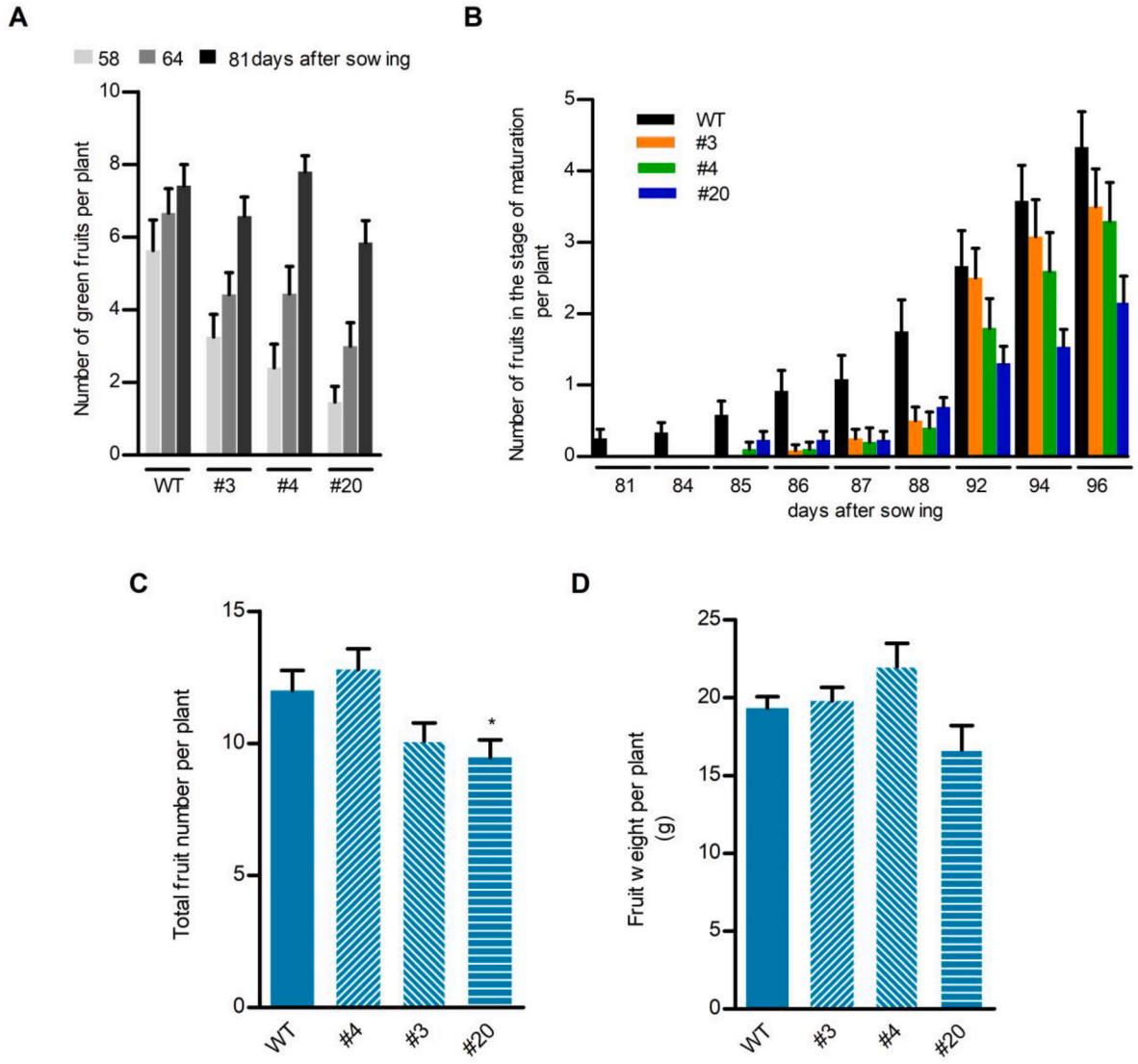

Fig. 3. Fruit growth and production in *Sl*BBX16OE plants. (A) Number of green fruits recorded from 58 to 81 days after sowing. (B) Number of fruits in the stage of maturation. (C) Total fruit number per plant recorded 110 days after sowing. (D) Average fruit weight. The values reported are means ± SE (n = 10–13). Student's t-test was used for the statistical analysis (*P < 0.05).

2020). TCMP-2 is specifically expressed in reproductive organs (Cavallini et al., 2011; Molesini et al., 2018) and, when overexpressed in flower buds, caused alteration in flowering pattern and early fruit setting in the processing tomato UC82, a determinate cultivar (Molesini et al., 2018, 2020). When *TCMP-2* is globally overexpressed (i.e., using the *CaMV35S* promoter) in the cultivar MicroTom, we have observed an anticipated formation of the primary inflorescence and a reduction in plant height, suggesting an accentuated determinate habit (Fig. S3). These data suggest that in both cultivars (UC82 and MicroTom), TCMP-2 is involved in the regulation of the flowering process. We use MicroTom to study whether *Sl*BBX16 and *Sl*BBX17 can play a role in reproductive development.

The microProteins *Sl*BBX16 and *Sl*BBX17 are highly homologous, with 46% identity (Fig. S1A), and belong to the group V BBX, which comprehends 5 members. The two genes are both expressed in the ovary and young fruit and co-expressed with *TCMP-2* (Figs. S4B and S5A, Cavallini et al., 2011; Molesini et al., 2020). Furthermore, Y2H analysis suggests that *Sl*BBX17 and *Sl*BBX16 share a common interacting partner, TCMP-2 (Fig. S5B and Molesini et al., 2020). Since previous studies demonstrated potential functional redundancy among *Sl*BBXs (Xu et al., 2022) and considering the high homology between *Sl*BBX16 and *Sl*BBX17, we focused our study on overexpressing lines to potentially dissect their contribution to tomato reproductive development.

3.2.1. *Overexpression of SlBBX16 alters fruit set and delays fruit growth in MicroTom*

We phenotypically characterized three *SlBBX16* overexpressing (*Sl*BBX16OE) lines #3, #4, and #20 (Fig. S4A). No changes in the number of leaves before the first inflorescence were observed compared to the WT plants (Fig. 2A), while the shoot height at the first inflorescence was significantly reduced only in the *Sl*BBX16OE #3 (Fig. 2B). These data suggest that flowering transition was not greatly affected by the *SlBBX16* overexpression. In addition, the number of flowers measured in the first three inflorescences was comparable in the WT and *Sl*BBX16OE lines (Fig. 2C). However, fruit set, calculated as a percentage of number of fruits over number of flowers in the first three inflorescences, was significantly lower in the *Sl*BBX16OE lines than in the WT plants (Fig. 2D and E). We have also detected a delay in the initial fruit formation and the onset of maturation assessed as number of fruits at the green stage (Fig. 3A) and number of fruits at the breaker stage or in maturation (Fig. 3B) overtime, respectively.

Considering the total fruit production harvested at 110 days after

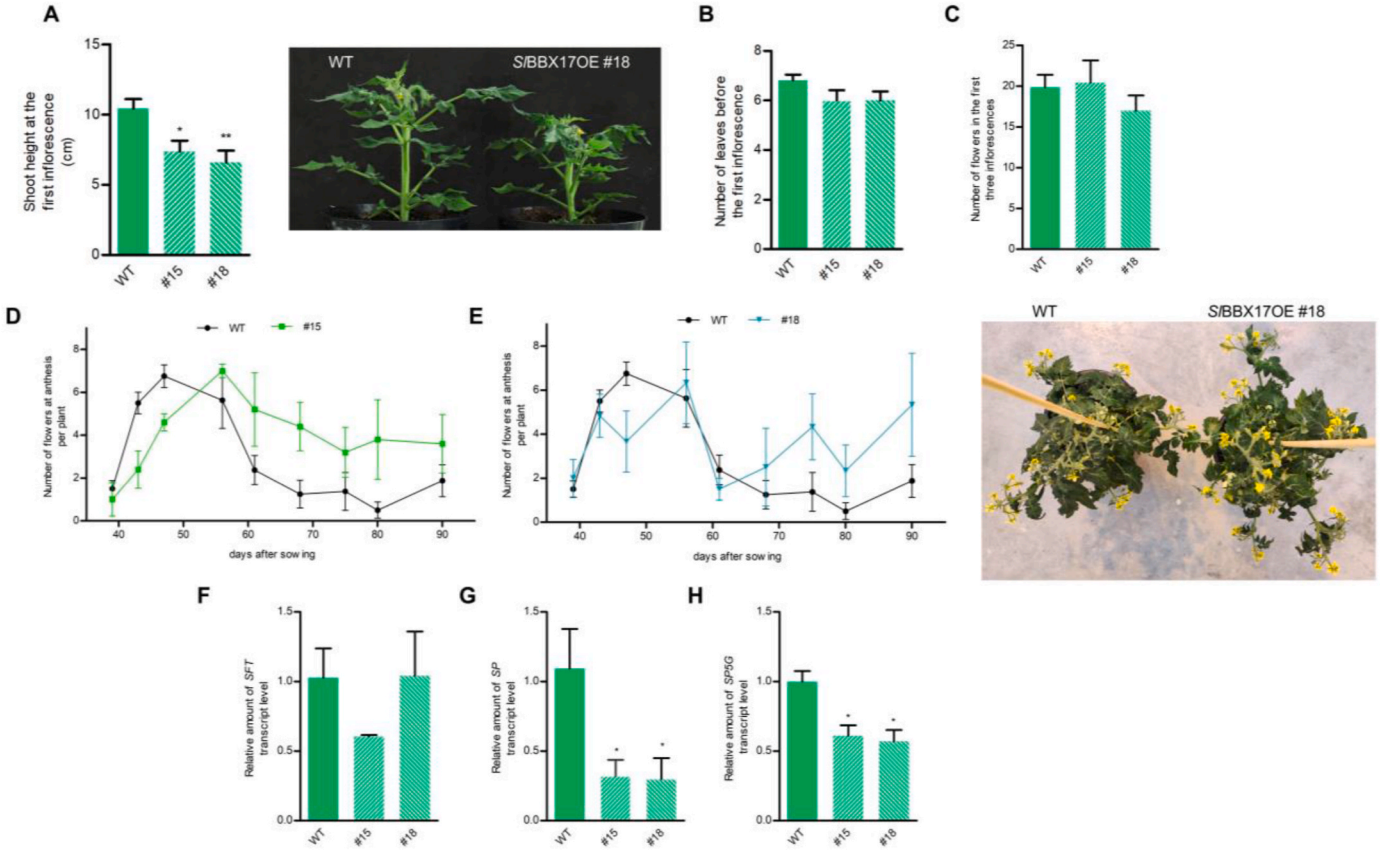

Fig. 4. Transition to flowering of *Sl*BBX17OE plants. (A) Shoot height at the first inflorescence. (B) Number of leaves before the first inflorescence. (C) Number of flowers in the first three inflorescences. (D and E) Number of flowers at anthesis per plant from 39 to 90 days after sowing. The values reported are means ± SE (n = 5–8). Phenotypical aspect of WT and *Sl*BBX17OE #18 at 85 days after sowing (panel E, on the right). (F–H) Expression level of *SFT*, *SP,* and *SP5G* of WT and *Sl*BBX17OE plants. Values are means ± SE of three biological replicates. Student's t-test was used for the statistical analysis (*P < 0.05; **P < 0.01).

sowing, in the *Sl*BBX16OE lines, there was a partial compensation of the fruit set impairment in the first three inflorescences (Fig. 3C). The weight of WT and transgenic fruits was similar (Fig. 3D), as was the percentage of red and green fruits (data not shown).

3.2.2. Overexpression of SlBBX17 prolongs flowering and reduces ripe fruit production in MicroTom

We investigated the phenotypic consequences of *SlBBX17* overexpression in MicroTom by comparing two independent *Sl*BBX17OE transgenic lines (#15 and #18) to WT plants (Fig. S5C). Differently from what was previously reported by overexpressing *SlBBX17* in the indeterminate cultivar Ailsa Craig (Xu et al., 2022), we did not observe retardation in vegetative growth and a reduction in leaf size (data not shown). However, because of the stunted growth of MicroTom, it is likely that these growth effects were masked by other mutations. In agreement with Xu and collaborators (2022), *Sl*BBX17OE plants showed moderate resistance to high temperatures (Fig. S5D). When we monitored the transition from vegetative to reproductive development in *Sl*BBX17OE plants, we observed a reduction in the height of the shoot bearing the primary inflorescence compared to WT plants, but no changes in the number of leaves before the first inflorescence (Fig. 4A and B).

The number of flowers in the first three inflorescences was similar in the WT and transgenic lines (Fig. 4C), but when recording the number of flowers at anthesis over time, we noticed a different trend in the WT and transgenic plants (Fig. 4D and E). In the WT plants, the number of open flowers increased from 39 to 47 days after sowing (das) but decreased sharply thereafter. In the transgenic plants, we observed a tendency to persist in producing flowers over time: in line #15 the number of flowers was higher than in WT from 61 das onwards and in line #18 this effect was detected from 75 das onwards. Given the differences in the duration of flowering between WT and *Sl*BBX17OE plants (Fig. 4D and E), we analysed the expression of *SFT*, the ortholog of *FT*, and the flowering inhibitors, *SP* and *SP5G*. The dynamic ratio between florigen and antiflorigens regulates shoot termination in tomato, which is associated with flowering (Lifschitz et al., 2006; Cao et al., 2016). The expression of *SFT* did not vary, whereas the expression of *SP* and *SP5G* was reduced in the transgenic plants compared to WT (Fig. 4F–H). This suggests that the prolonged flowering observed in *Sl*BBX17OE might be associated with a weaker antagonistic effect of SP on SFT. Tomato is a day-neutral plant and the mechanism controlling the flowering process in this species is not fully elucidated. In this regard, the biological functions of CO-like (COL) genes in tomato remain elusive. Recently, *Sl*COL1 (Solyc02g089540) was shown to interact with the *SFT* promoter, repressing its transcription and consequently inhibiting the flowering transition (Cui et al., 2022). Considering that miP1a/b interacts with *At*CO, regulating its activity, we tested by Y2H the interaction between *Sl*BBX16 and *Sl*BBX17 and *Sl*COL1. The absence of interaction between tomato BBX proteins and *Sl*COL1 would suggest that either *Sl*BBX16 and *Sl*BBX17 do not bind tightly to *Sl*COL1 under our experimental conditions or that their interaction requires additional factors that are absent in yeast (Fig. S2).

We then evaluated the fruiting process in the *Sl*BBX17OE lines assessing fruit set and fruit productivity. The fruit set recorded in the first three inflorescences and the total number of fruits per plant evaluated 4 months after sowing were similar to WT plants (Fig. 5A and B).

However, the proportion of ripe fruits out of the total number of fruits in the transgenic lines was reduced (Fig. 5C and F). In addition,

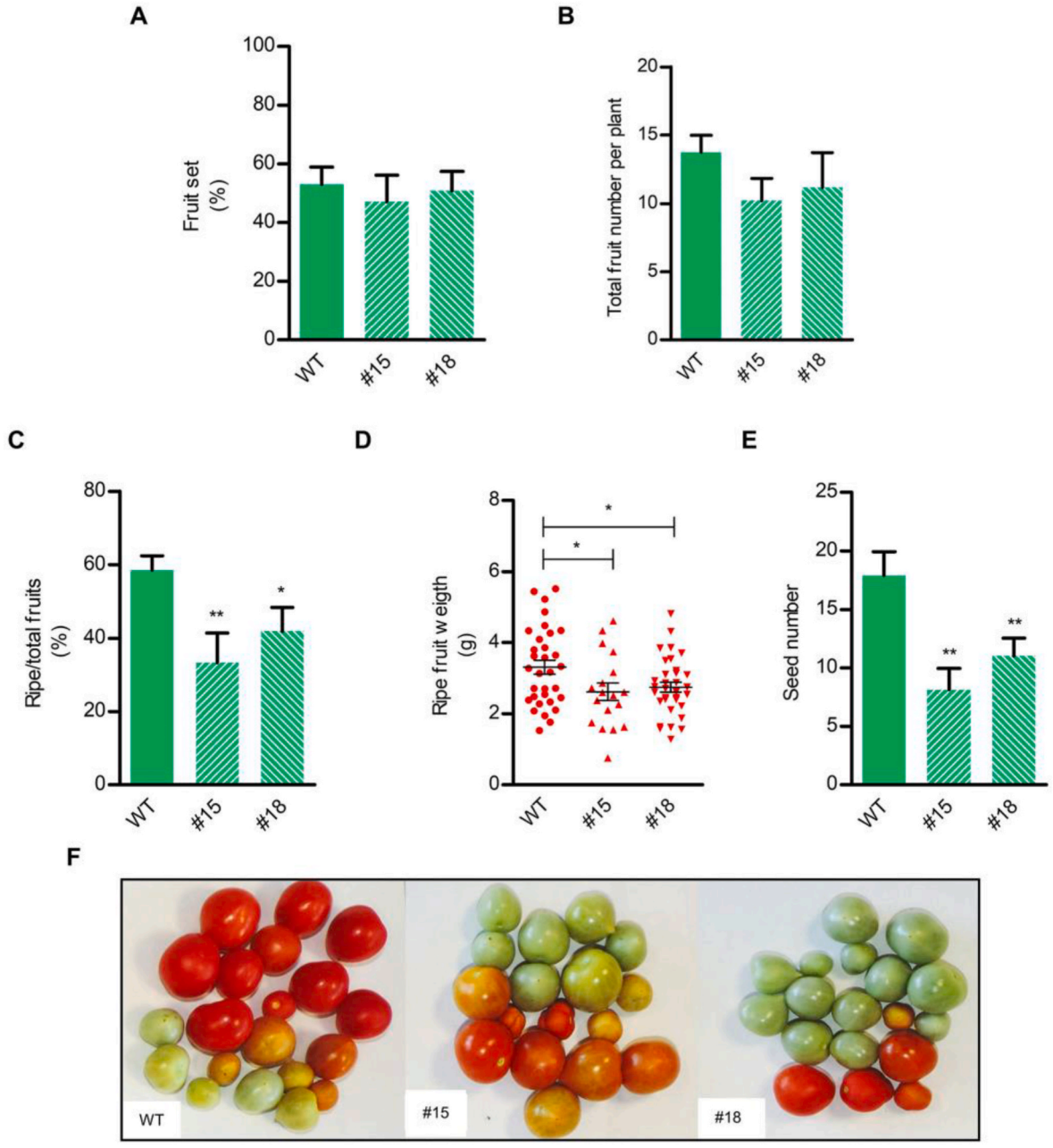

Fig. 5. Fruit growth and production in *Sl*BBX17OE plants. (A) Fruit set percentage of the first three inflorescences calculated as the percentage of number of fruits over number of flowers. (B) Total fruit number per plant, (C) percentage of ripe over total fruits collected 110 days after sowing (D) weight of ripe fruits and (E) number of seeds collected from ripe fruits. (F) Representative picture of fruit production. The values reported are means ± SE (n 8–14 for panels A, B and C; n = 18–34 for panels D and E). Student's t-test was used for the statistical analysis (*P < 0.05; **P < 0.01).

ripe fruits were smaller and contained fewer seeds (Fig. 5D and E). We have also observed a different weight distribution of green fruits with a decreasing trend in *Sl*BBX17OE plants (Fig. S6).

3.3. *SlBBX16 and SlBBX17 can affect the expression of GA-related genes*

To investigate if the different effects of *Sl*BBX16 and *Sl*BBX17 actions on fruit development were associated with perturbation of hormone homeostasis, we examined in *Sl*BBX16OE and *Sl*BBX17OE immature green fruits, a phase of active growth and preparation for ripening, the expression of a set of genes implicated in ethylene, abscisic acid (ABA), and gibberellin (GA) metabolism. Aminocyclopropane-1-carboxylic acid synthase 1A (ACS1A) and ACS6 encode enzymes of the ethylene biosynthetic system 1 that triggers the transition to the breaker stage in green fruit (Barry et al., 2000). Their expression did not vary in *Sl*BBX16OE and *Sl*BBX17OE fruits (Fig. 6A, B, E, F), suggesting that ethylene biosynthesis at this stage is similar to that in WT fruits. To test a possible alteration of ABA metabolism, we analysed the expression of 9-cis-epoxycarotenoid dioxygenase (*SlNCED-1*), the key determinant of ABA synthesis, and *SlZFP2*, a zinc finger transcription factor involved in the crosstalk between ABA and ethylene in the regulation of fruit ripening in tomato (Weng et al., 2015). The expression of *SlNCED-1* increased in two (#3 and #4) out of the three *Sl*BBX16OE lines, while it remained unaltered in *Sl*BBX17OE fruits compared to WT (Fig. 6D and H). The transcript level of *SlZFP2* was unchanged in *Sl*BBX16OE and *Sl*BBX17OE fruits (Fig. 6C and G).

To assess possible changes in GA metabolism and signaling, we examined the expression of several key genes involved in GA

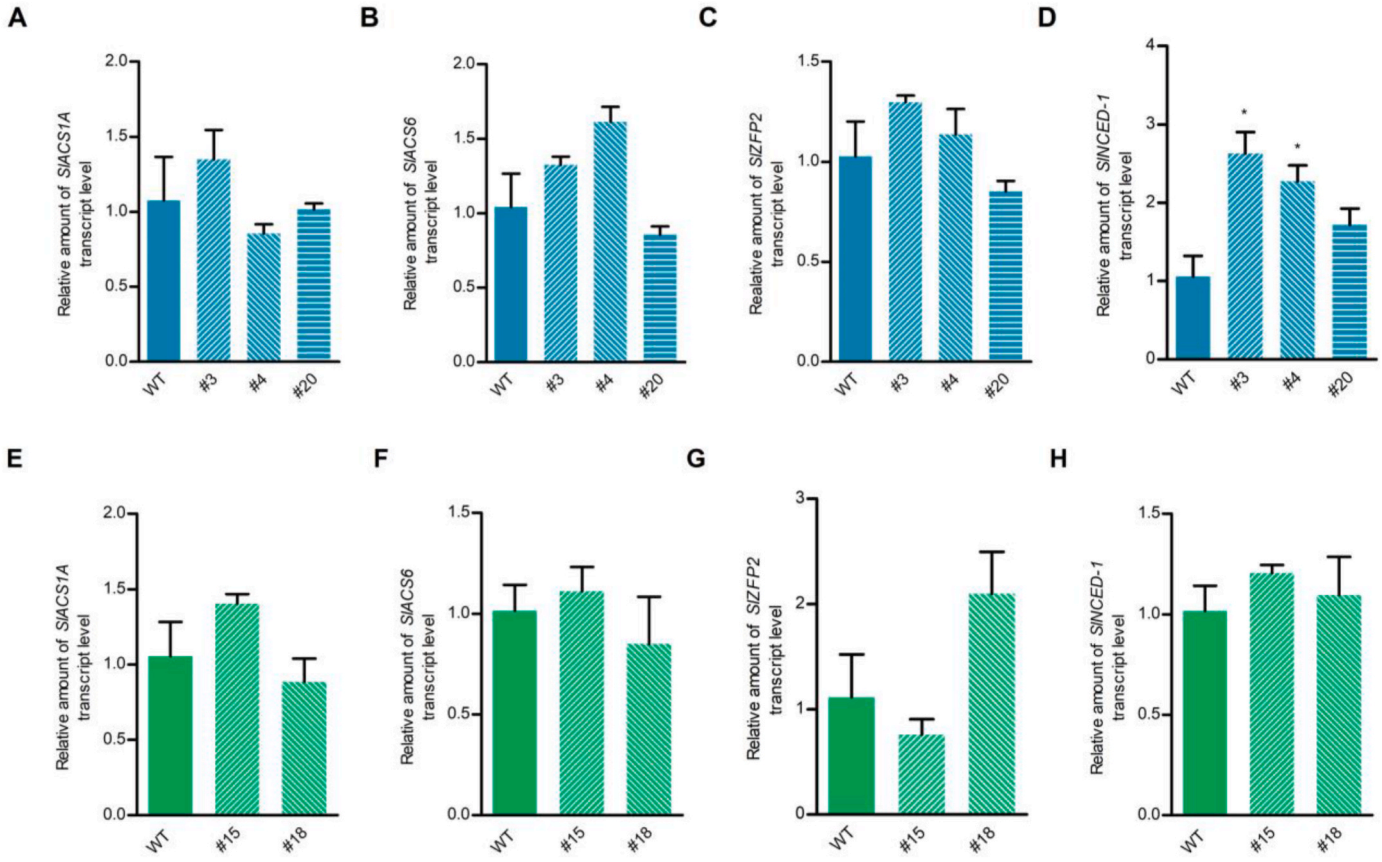

Fig. 6. Expression of genes implicated in ethylene and abscisic acid metabolism. Transcript levels of *ACS1A*, *ACS6*, *ZFP2*, and *NCED-1* in WT and *Sl*BBX16OE (A–D) and *Sl*BBX17OE (E–H) green fruits. Values are means ± SE of three biological replicates. Student's t-test was used for the statistical analysis (*P < 0.05).

biosynthesis and catabolism, such as *SlGA20 oxidase 1* (*SlGA20ox1), SlGA20ox2,* and *GA2ox4*, and in GA perception and sensitivity, such as *DELLA*, *GA-INSENSITIVE DWARF1b* (*GID1b)*, and *GA Stimulated Transcript 1* (*GAST1*).

In the three *Sl*BBX16OE lines, *SlGA20ox2* displayed a consistent downregulation, whereas *SlGA2ox4* expression remained unchanged (Fig. 7A). Furthermore, the expression of *GID1b* decreased in all the *Sl*BBX16OE lines, while no changes were observed for *DELLA* and *GAST1* (Fig. 7B). In both *Sl*BBX17OE lines, *SlGA20ox1* was consistently reduced, whereas *SlGA20ox2* decreased in line #15 and *SlGA2ox4* increased in line #18 (Fig. 7C). In addition, both lines showed a decline in the expression of *GID1b* and an upregulation of *GAST1*, whereas the transcript level of DELLA remained unaltered (Fig. 7D). Quantification of active GA (GA_1 and GA_4) in green fruits revealed a significant increase in *Sl*BBX17OE line #18, while GA levels in *Sl*BBX16OE line #3 were unchanged (Fig. 7E). Furthermore, it is worth noticing that treatment with exogenous GA_3 induced the expression of *Sl*BBX16 and *Sl*BBX17 (Fig. 7F and G). Despite the complexity of the mechanisms that regulate active GA levels, including GA metabolism, transport, and signaling (Hedden, 2020), the observed changes in the expression profiles of GA-related genes suggest that overexpression of *Sl*BBX16 and *Sl*BBX17 causes a perturbation of GA response in tomato fruits. In this regard, the upregulation of *GAST1* and the increase in active GA content in *Sl*BBX17OE fruits are consistent with the delay in ripening (Su et al., 2023). Indeed, *Sl*BBX17OE lines displayed a reduced percentage of ripe fruits (Fig. 5C) and a retarded maturation after injection of the fruits with exogenous GA_3 (Fig. S7).

4. Discussion

Plant microProteins are small polypeptides characterized by a single domain involved in protein-protein interaction. From an evolutionary point of view, miPs can originate from larger proteins by duplication and domain loss (*trans*-miPs) or derive from alternative splicing or alternative transcription start and stop sites (*cis*-miPs) (Eguen et al., 2015; Kushwaha et al., 2022). Their capacity to interact with homologous proteins of larger size (homotypic interaction) is at the basis of their activity as posttranslational regulators of protein complexes. Besides homotypic, plant miPs can engage also in heterotypic interactions with evolutionary unrelated proteins, widening the regulatory role of these proteins (Bhati et al., 2021).

Arabidopsis miP1a and miP1b are microProteins belonging to subclass V of the BBX family. They play a role in photomorphogenic development and control of flowering time. Both miP1a and miP1b participate in seedling growth arrest after germination under stress conditions by interacting heterotypically with ABA-insensitive 5 (ABI5) and stabilizing it (Singh and Datta, 2023). During the transition from dark to light conditions, miP1a and miP1b inhibit the oligomerization of PHYTOCHROME-INTERACTING FACTORs (PIFs) and ETHYLENE-INSENSITIVE 3 (EIN3) (Wu et al., 2020). A homotypic interaction between miP1a/b and *At*CO regulates florigen *FT* transcription; the strong delay in flowering observed by overexpressing miP1a/b in *Arabidopsis* is caused by the recruitment of TOPLESS to miP1a/b-CO complex resulting in inhibition of *FT* transcription (Graeff et al., 2016).

In tomato, *Sl*BBX16 and *Sl*BBX17 microProteins are the closest homologs of miP1a/b. A study by Xu and collaborators (2022) reported that *SlBBX17* overexpression in an indeterminate tomato cultivar (i.e., Ailsa Craig) resulted in increased heat tolerance and growth retardation (Xu et al., 2022). More recently, *SlBBX17* was reported to participate also in the response to cold stress (Song et al., 2023). The effects of *SlBBX17* overexpression on reproductive development were not

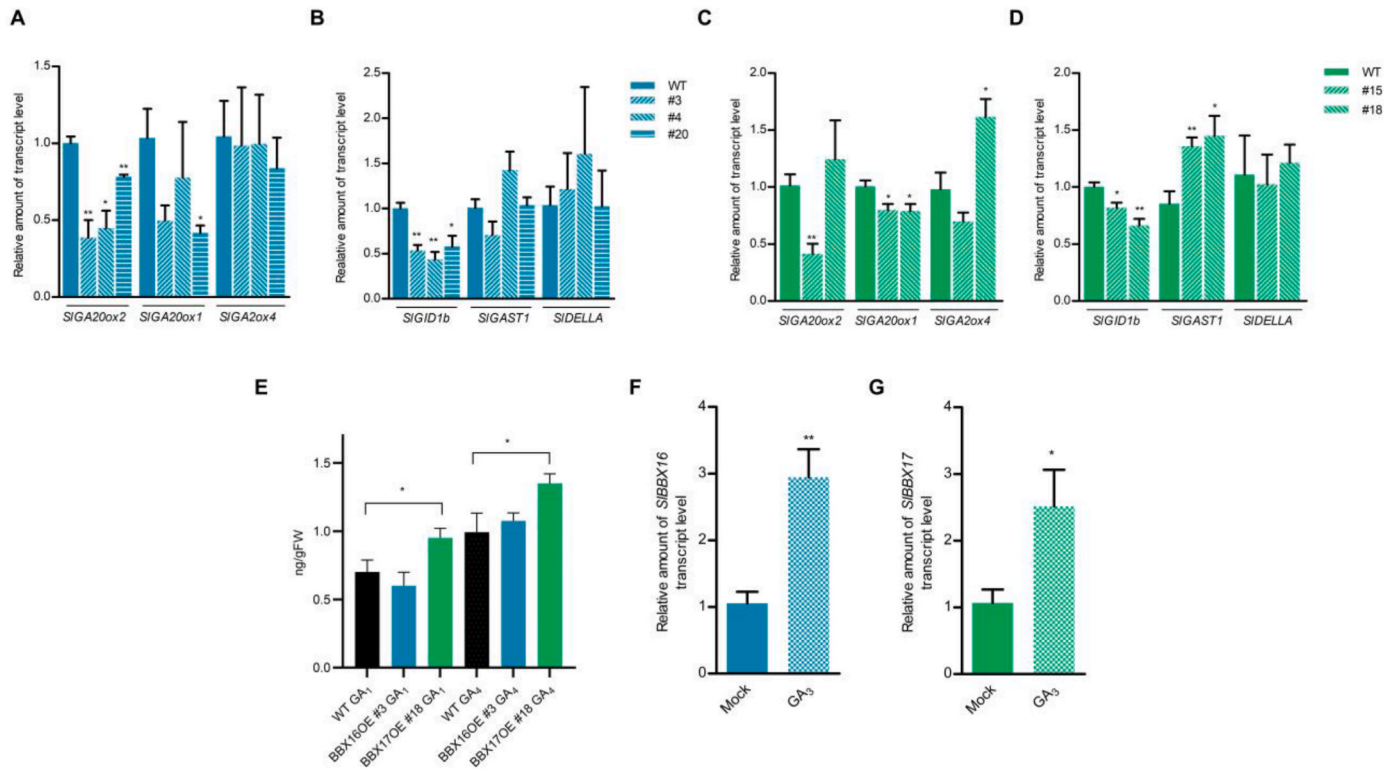

Fig. 7. Expression analysis of genes implicated in GA biosynthesis (*GA20ox2, GA20ox1*) and catabolism (*GA2ox4*) in WT, *Sl*BBX16OE (A) and *Sl*BBX17OE (C) fruits. Expression analysis of genes implicated in GA perception/signaling (*GID1b*, DELLA) and sensitivity (*GAST1*) in WT, *Sl*BBX16OE (B) and *Sl*BBX17OE (D) fruits. (E) Bioactive GAs (GA_1 and GA_4) content in immature green fruits of *Sl*BBX16OE #3 and *Sl*BBX17OE #18 in comparison with WT. Response of *Sl*BBX16 (F) and *Sl*BBX17 (G) to GA_3. Relative expression of *SlBBX16* and *SlBBX17* was evaluated in shoots of seedlings treated for 24 h with 5 μM GA_3 in comparison with mock-treated ones. Values are means ± SE (n = 3–6). Student's t-test was used for the statistical analysis (*P < 0.05; **P < 0.01).

investigated in those studies. The first hints on the implication of *Sl*BBX17 and *Sl*BBX16 in reproductive development derive from the evidence that *Sl*BBX16 and *Sl*BBX17 undergo heterotypic interactions with a tomato cystine-knot peptide, TCMP-2, which plays a role in both flowering pattern and fruit development (this work and Molesini et al., 2020). In the tomato cultivar UC82, overexpression of *TCMP-2* in flower buds promoted the termination of sympodial units (Molesini et al., 2020) and global overexpression in MicroTom resulted in the early appearance of the first inflorescence (this work). In addition, ectopic overexpression of *TCMP-2* anticipated flowering in *Arabidopsis* and in both tomato and *Arabidopsis*, TCMP-2 induced the transcription of florigen (Molesini et al., 2020). To investigate if TCMP-2 interacting partners play a role in flowering, *SlBBX16* and *SlBBX17* were overexpressed in *Arabidopsis* WT Col-0 and miP1a/b double KO mutant and in tomato. *Arabidopsis Sl*BBX16OE and *Sl*BBX17OE plants showed a weak delay in flowering, which is evident for *Sl*BBX16 only when overexpressed in a background depleted in miP1a/b function. This observation suggests that the mechanism of action of *Sl*BBX16 differs somewhat from that of *Sl*BBX17. The incomplete functional redundancy between the two proteins is confirmed by the fact that overexpression of *Sl*BBX16 in tomato did not cause appreciable alterations in flowering, whereas overexpression of *Sl*BBX17 resulted in protracted flower production. This latter effect is caused by a reduced expression of the antiflorigens, *SP* and *SP5G*. Thus, overexpression of *TCMP-2* and its partners produced contrasting effects on flowering, suggesting an antagonistic interaction. The evidence that both TCMP-2 and *Sl*BBX16/17 showed no direct interaction with *At*CO and *Sl*COL1 (this work and Molesini et al., 2020) highlights the differences in the flowering control system between *Arabidopsis* and tomato. In this regard, *Sl*COL1 has been shown to bind the *SFT* promoter and negatively regulate its expression (Cui et al., 2022). Based on our results, TCMP-2 and *Sl*BBX17 activity in the tomato flowering process appeared independent of the *Sl*COL1/SFT regulatory module. We cannot exclude that *Sl*BBX17 can interact with other members of the *Sl*COL family.

This study demonstrates that *Sl*BBX16 and *Sl*BBX17 also participate in the regulation of fruit growth and maturation. In a recent paper, Lira and collaborators evaluated the transcript profiles of *SlBBX16* and *SlBBX17* in tomato fruits at different stages of development, starting from immature green up to 5 days after the breaker stage (Lira et al., 2020). During the stages of green fruit growth, the expression of *SlBBX16* progressively decreased (Lira et al., 2020). This expression pattern might indicate that during the period of fruit enlargement, *Sl*BBX16 activity should remain low, an observation consistent with the phenotype of *Sl*BBX16OE plants, in which fruit growth is delayed from the early green phases until the breaker stage. It is plausible that *Sl*BBX16 is one of the factors that restricts ovary expansion; therefore, its progressively reduced expression would be necessary for optimal fruit growth. The reduced fruit set observed in the first three inflorescences of *Sl*BBX16OE plants would support this hypothesis. The fact that the increased expression of *TCMP-2* resulted in anticipated fruit development would strengthen the assumption of a negative interaction between TCMP-2 and *Sl*BBX16.

The expression of *SlBBX17* is not sharply modulated during fruit development as for *SlBBX16* (Lira et al., 2020); its transcript level increases weakly from immature green to the breaker stage and then declines shortly (Lira et al., 2020). In the present work, the principal effect of *SlBBX17* overexpression was observed at the ripening stage. The percentage of ripe to total fruits was markedly reduced, suggesting that the excess of this microProtein at the maturation phase hinders this process. The reduced weight of ripe *Sl*BBX17OE fruits and the similar tendency observed for green fruits indicate that *Sl*BBX17 is also involved in fruit enlargement.

The two microProteins seem to contribute to regulating fruit development at different stages, but both lead in young fruits, when

overexpressed, to modification in the transcript levels of genes related to GA metabolism and signaling. Remarkably, both *SlBBX16* and *SlBBX17* are GA responsive (this work and Chu et al., 2016; Lira et al., 2020). During tomato fruit development, GA presents a bimodal accumulation pattern that reflects their contribution in different phases of this process. A high GA level in flowers promotes fruit set and in young fruits induces growth through cell expansion, while GA level decreases during fruit ripening (Li et al., 2019). Consistently, exogenous application of GA_3 to mature green fruit delayed fruit ripening (Li et al., 2019).

In *Sl*BBX16OE fruits at the immature green stage, we observed a downregulation of *GA20ox2* and *GID1b* but not a modification in bioactive GA content. These changes cannot be easily associated with the retardation of early fruit growth in *Sl*BBX16OE plants, thus, it would be crucial to further decipher both interactors and targets of this microProtein. On the other hand, the delayed ripening of *Sl*BBX17OE fruits is consistent with both the increased content of GA_1 and GA_4, and the induced expression of *GAST1,* which is a molecular marker of changes in active GA level and a repressor of fruit ripening (Li et al., 2019; Su et al., 2023). In addition, the downregulation of *SlGA20ox1* and *GID1b* might be a consequence of GA feedback regulation. In fact, GA homeostasis is controlled by the GA signaling pathway, involving DELLA proteins, negative regulators degraded in the presence of GA. It has been demonstrated in *Arabidopsis* that DELLA, at low concentrations of GA, induces the expression of *GA20ox2* and *GID1* by forming a complex with the transcription factor GAI-ASSOCIATED FACTOR 1 (GAF1) (Fukazawa et al., 2014). Degradation of DELLA allows the interaction of GAF1 with TOPLESS RELATED (TPR) proteins inhibiting target genes, such as *GA20ox2* and *GID1b* (Fukazawa et al., 2014).

It will be critical for future research to unravel the regulatory functions of these tomato microProteins in the reproductive process, investigating whether they may be part of a multiprotein complex together with TCMP-2. Ultimately, a better understanding of the mechanism of action by which these microProteins alter GA responses will provide opportunities for their potential application in tomato breeding to match the reproductive phases with changes in climate conditions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.108873.

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