

Overexpression of *SIMBP22* in Tomato Affects Flower Morphology, Fruit Set and Development

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Abstract

MADS-domain transcription factors have been clarified as key regulators involved in proper flower and fruit development in angiosperms. B_s genes, as members of the MADS-box subfamily, have been suggested to play an important role during the evolution of the reproductive organs in seed plants. Our knowledge about their effects on reproductive development in fruit crops like tomato (*Solanum lycopersicum*), however, is still unclear. Here, we found that the overexpression of *SIMBP22* (*SIMBP22*-OE) resulted in considerable alterations regarding floral morphology, and affected the expression levels of several floral homeotic genes. Further analysis by yeast-two-hybrid assays demonstrated that *SIMBP22* could form dimers with class A protein MACROCALYX (MC) and with SEPALLATA (SEP) floral homeotic proteins TM5 and TM29, respectively. In addition, pollen viability and cross-fertilization assays suggested that the defect in female reproductive development was responsible for infertility phenotype observed in the strong overexpression transgenic plants. The mild overexpression transgenic fruits were reduced in size, as a result of reduced cell expansion, rather than impaired cell division. Additionally, overexpression of *SIMBP22* in tomato not only affected proanthocyanidin (PA) accumulation but also altered seed dormancy. Taken together, these findings may provide new insights into the knowledge of B_s MADS-box genes in flower and fruit development in tomato.

Key Message

Overexpression of B_{sister} (B_s) MADS-box gene *SIMBP22* impacts tomato reproductive development, including flower morphology, fruit set, fruit size and seed pigmentation.

Introduction

Reproductive development of higher plants entails a series of biological processes, including floral meristem determination, floral bud generation, fruit development and ripening, all aimed at promoting seed formation and dispersal to ensure progeny survival. MADS-box proteins, have played prominent roles in the transcriptional modulation of the floral organ specification and reproductive development, and are one of the most thoroughly studied gene family members in plants (Gramzow and Theissen 2010; Ng and Yanofsky 2000).

Floral development is a complex biological process and highly regulated by both the genetic background of plants and environmental factors (Fornara, et al. 2010). Previous studies have revealed that floral homeotic genes determining reproductive floral organ identities can be well understood via ABCDE model. (Coen and Meyerowitz 1991; Colombo, et al. 1995; Theissen 2001; Theissen and Saedler 2001).

Interestingly, all genes thus far identified in this model, except for *APETALA2* (*AP2*), encode MIKC^C-type proteins and belong to MADS-box transcription factor family (Parenicova, et al. 2003). With the deepening of research, more and more MADS-box genes have been identified and characterized as key regulators of tomato flower development. For instance, tomato class A gene *MC* is involved in the inflorescence determinacy and the sepal development (Vrebalov, et al. 2002). Overexpression of *FYFL* in

tomato presents longer sepals than wild-type (Xie, et al. 2014). Tomato class C gene *TOMATO AGAMOUS 1 (TAG1)*, as the cognate homologs of Arabidopsis *AGAMOUS (AG)*, participants in the regulation of stamen and carpel development as well as floral meristem determinacy (Pan, et al. 2010; Pnueli, et al. 1994). *TOMATO AGAMOUS-LIKE1 (TAGL1)*, is the tomato ortholog of duplicated *SHATTERPROOF* of Arabidopsis, and also is the most closely related gene to *TAG1*, playing a key role in regulating carpels development (Vrebalov, et al. 2009). Tomato plants with up-regulated mRNA level of D-class MADS-box gene, *Sl-AGL11*, display carpel-like sepals (Huang, et al. 2017). *SIMBP3* is the most closely related paralog of *Sl-AGL11*, and is notably expressed in the pistils. Silencing of *SIMBP3* affects the development of seeds/placenta, suggesting that this gene specifies carpel/ovule identity (Zhang, et al. 2019). Two E-class MADS-box genes, *TM5* and *TM29*, have predominant functions in the development of floral organs and the determination of floral meristem identity in tomato.

Generally, early fruit development undergoes three major phases, namely, fruit set, cell division and cell expansion. Fruit development involves complex spatial and temporal regulation by the interplay of numerous biotic and abiotic factors, such as plant hormones, transcription factors, elongation factors, microRNA, RNA-binding proteins, ubiquitin-proteasome and so on (Hussain, et al. 2020). In addition to researches describing the influence of MADS-box proteins on flower development, there have also been numerous studies highlighting the roles of MADS-box transcription factors in mediating various fruit morphologies, ripening and seed dispersal. Besides the role of tomato *SHATTERPROOF 1* and *2 (SHP1, 2)* ortholog *TAGL1* in floral organ identities, this gene also functions in the fruit expansion and ripening process. *TAG1* and *TAGL1* are paralogous genes, the *TAG1* silenced plants display smaller fruits than wild-type, which may be related to the reduction of pericarp thickness (Gimenez, et al. 2016; Vrebalov, et al. 2009). Transgenic plants with reduced tomato *SEP1, 2* ortholog *TM29* expression levels show phenotype with parthenocarpic fruits (Ampomah-Dwamena, et al. 2002). Class D gene *Sl-AGL11*, a close paralog of *SIMBP3*, is involved in the regulation of fruit quality and productivity (Huang, et al. 2017).

The phylogenetic sister clade of the class B genes has been termed B_{sister} (B_s) (Becker, et al. 2002). Relatively few members of this subfamily involved in the plant vegetative and reproductive growth have so far been characterized in different angiosperm species. It has been considered that B_s genes *make a valuable contribution* to the regulation of reproductive development in seed plants. For instance, the B_s MADS-box transcription factor, *GORDITA (GOA)*, controls fruit size largely by modulating cell expansion in Arabidopsis (Prasad, et al. 2010). *ABS/TT16/AGL32*, the closest relative of *GOA*, is involved in the regulation of seed coat pigmentation and proanthocyanidin (PA) accumulation in the inner endothelial cell of the developing seeds in Arabidopsis (de Folter, et al. 2006; Nesi, et al. 2002; Xu, et al. 2017). It has been shown that *ABS* acts redundantly in the formation of endothelium with the D-class MADS-box protein *SEEDSTICK (STK)*. The very few seeds observed in the Arabidopsis *abs stk* double mutant caused by the reduction of the number of fertilized ovules and the seed abortions (Mizzotti, et al. 2012). Similarly, *FLORAL BINDING PROTEIN 24 (FBP24)* is necessary for proper endothelium development in petunia (*Petunia hybrida*). Nevertheless, a mutant complementation experiment demonstrates that *FBP24* fails to replace *ABS/TT16*, suggesting that there are functional conservation

and divergence of the supposed orthologous genes in different angiosperm species (Becker, et al. 2002). *OsMADS30* T-DNA insertion plants display the alterations of plant size and architecture in rice, indicating that *OsMADS30* may have evolved a new function and therefore is not a canonical B_s gene (Schilling, et al. 2015).

Although we previously identified a tomato B_s gene *SIMBP22*, homologous to Arabidopsis *ABS/TT16* and petunia *FBP24*, participated in regulating tomato growth and tolerance to drought stress (Li, et al. 2020), its role in *reproductive development* has not been fully explored. In this study, we found that *transgenic tomato plants overexpressing SIMBP22 exhibited phenotypes related to defects in floral architecture, fruit set and development*. Moreover, *the underlying causes for these phenotypes were respectively analyzed at the morphological, statistical and molecular levels*. Our data further expand the understanding of the functions of B_s MADS-box proteins in the regulation of plant *reproductive development*.

Materials And Methods

Plant materials and growth conditions

Tomato (*Solanum lycopersicum* Mill. cv. Ailsa Craig) wild-type (WT) and transgenic plants were grown under normal greenhouse conditions (16-h-day/8-h-night cycle, 25°C/18°C day/night temperature, and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity). For gene expression analysis, flowers were harvested at different developmental stages: -4D (4 d ahead of anthesis), -2D (2 d ahead of anthesis), anthesis and 2D (2 d after anthesis), and four-whorl mature floral organs (sepals, petals, stamens and pistils) were harvested at the anthesis stage. All samples were collected and promptly frozen in liquid nitrogen and then stored at -80°C until required.

Vector construction and plant transformation

For the construction of the *SIMBP22*-overexpressing (*SIMBP22*-OE) vector, the *SIMBP22* full-length coding region was amplified by PCR with primers *SIMBP22*-F and *SIMBP22*-R, adding the *Xba* I and *Sac* I site to the 5' end and 3' end, respectively (Supplementary Table S1). The amplified *SIMBP22* products were digested with *Xba* I and *Sac* I and then ligated into the plant binary vector pBI121 placed under the control of CaMV 35S promoter. The resulting binary vectors were transferred into *S. lycopersicum* variety AC⁺⁺ cotyledons, according to the transformation and regeneration methods as previously reported (Chen, et al. 2004).

Gene expression analysis

Total RNA was isolated using RNAiso Plus (Takara). The cDNA was synthesized using M-MLV Reverse Transcriptase Kit (Promega). Gene expression levels in different organs were evaluated by quantitative real-time PCR (qRT-PCR) using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). *SICAC* (Solyc08g006960), a tomato housekeeping gene, was used as an internal control (Exposito-Rodriguez, et

al. 2008). The analysis of relative expression levels was conducted using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All primers for qRT-PCR are presented in Supplementary Table S1.

Yeast two-hybrid assays

The ORFs of *SIMBP22*, *MC*, *TAGL1*, *SIMBP3*, *TM5* and *TM29* were amplified by PCR using primers (Supplementary Table S1). The PCR fragments of *SIMBP22* were cloned into the pGBKT7 vector to generate a bait construct BD-MBP22, and *MC*, *TAGL1*, *SIMBP3*, *TM5* and *TM29* were linked into pGADT7 vector to obtain prey constructs, namely, AD-MC, AD-TAGL1, AD-SIMBP3, AD-TM5 and AD-TM29, respectively. Different combinations of bait and prey vectors were co-transformed into Y2Hgold. The yeast two-hybrid assays were conducted as described previously (Tang, et al. 2020). The experiments were repeated three times.

Pollen viability assay

Pollen viability assay was tested by TTC staining method described previously (Huang, et al. 2017). Briefly, soaked pollen grains of the fully opened flower from the wild-type and transgenic plants in a 0.1% 2,3,5-triphenyl-2 h-tetrazolium chloride (TTC) solution at room temperature for 15 min, and then, stained pollen grains were observed and photographed under the microscope (Nikon E100). The experiments were repeated three times.

Cross assay

A cross assay was conducted according to the method described in a previous report (Shen, et al. 2019). The anthers of the WT and three OE plants (lines 18, 2 and 17) were carefully removed at 2 days ahead of anthesis, and the emasculated flowers were labeled and bagged with small plastic bags to prevent natural pollination. Two days after emasculatation, mature pollen grains of newly opened flowers from the two OE lines, OE18 and OE2, were collected and brushed to the styles of the emasculated flowers from the WT plants. Meanwhile, we brushed mature pollen grains from the newly opened WT flowers to the styles of the emasculated flowers of the transgenic lines: OE 18, 2 and 17, respectively.

Histological analysis

Histological [analyses](#) of sepal at the anthesis stage and fruit pericarp at the breaker stage from the WT and *transgenic* plants were processed following the method as described in our previous report (Li, et al. 2020). The morphological observations of the paraffin sectioning were performed under a light microscope (Nikon E100). The number of cell layers and the mean mesocarp cell size were estimated according to our previous report (Zhu, et al. 2019).

Vanillin Assay and PA extraction

Vanillin staining of mature tomato seeds was carried out using vanillin reagent as previously described (Chen, et al. 2013; Debeaujon, et al. 2000). Briefly, fresh seeds of the three independent mild *transgenic*

OE lines and the WT plants at the 42d post anthesis (DPA) stage were harvested and soaked in a 1% vanillin solution at room temperature for 30 min. PAs of tomato seeds were determined by using the Vanillin-HCl method according to a previous report (Gao, et al. 2018; Mitsunaga, et al. 1998).

Seed germination assays

The seed germination assays were performed as previously described (Zhou, et al. 2019). In brief, the WT and T2 homozygous transgenic seeds were sown onto MS medium after surface sterilization, and then germinated in the dark at 25°C for 7 days. Seed germination rates were recorded on days 3, 5 and 7 respectively. The experiments were repeated three times.

Statistical analysis

Data presented in this report are means \pm standard deviation from three independent repeats. The significant difference was assessed using Student's t-test ($P < 0.05$).

Results

Expression patterns of *SIMBP22*

B_s MADS-box transcription factors have important regulatory functions during the evolution of the reproductive organs in seed plants (Becker, et al. 2002). Our previous report indicated that *SIMBP22* may play essential roles in tomato flowers, fruits and roots development based on its expression pattern analysis (Supplementary Fig. S1) (Li, et al. 2020). To further explore the potential functions of *SIMBP22* in tomato, we further evaluated its relative expression levels in flowers at different development stages and in four-whorl floral organs at the anthesis stage by qRT-PCR analysis. The results showed that the transcript abundances of *SIMBP22* were higher in four days ahead of anthesis and anthesis flowers than that in two days before and after anthesis flowers (Fig. 1a). In addition, *SIMBP22* transcripts were mainly abundant in the pistils of floral organs, consistent with other plant B_s MADS-box genes (Chen, et al. 2012) (Fig. 1b). These results hinted that *SIMBP22* may participate in the regulation of floral organ development, especially female reproductive organ development in tomato.

Overexpression of *SIMBP22* alters tomato flower morphology and affects floral organ identity genes

We successfully generated five independent transgenic OE lines that were used for further study (Supplementary Fig. S2) (Li, et al. 2020), and observed that all of these lines displayed aberrant characteristics related to reproductive parts. The most evident phenotype was that all the *SIMBP22* overexpression plants displayed smaller flowers, especially the strong overexpression transgenic lines OE18 and OE2 (Fig. 1c, d). The measurements of the lengths of the four types of floral organs (sepals, petals, stamens and pistils) indicated significant reductions in the strong overexpression transgenic plants than those in the wild-type plants (Fig. 1e). Of particular note, the strong overexpression transgenic sepals were *extremely abnormal in development, their color was a lighter green, the size was reduced by*

approximately 61% to 63% and then could not wrap the petals, when compared to the equivalent organs in the wild-type plants. Also, the petals of the *SIMBP22*-OE plants were more yellow than those of the WT, and had curly edges (Fig. 1c, d). To determine whether the light green sepal phenotype represented a change in total chlorophyll content, we extracted chlorophyll from sepals of fully opened flowers and observed that the WT plants possessed higher chlorophyll levels compared with the strong *SIMBP22*-OE lines (Fig. 1f). Furthermore, the expression levels of genes related to chlorophyll biosynthesis and degradation, *CHLH*, *CHLM*, *CAO1* and *SGR1* (Hu, et al. 2011), were examined in sepals of both the wild-type and *SIMBP22*-OE transgenic plants by qRT-PCR analysis. The results showed that these genes were dramatically down-regulated in the transgenic plants (Supplementary Fig. S3).

MADS-box family genes, the major members of plant floral organ identity genes, have a central role in the regulation of flower development. Based on the functions of MADS-box genes in floral organs, these genes are subdivided into five classes according to the ABCDE model (Deng, et al. 2012; Smaczniak, et al. 2012; Theissen 2001). Considering that overexpressing *SIMBP22* caused defects in flower morphology, the expression levels of floral organ identity genes were assessed by qRT-PCR. As shown in Fig. 2a, the mRNA level of *MC*, one of the A-class genes and plays an essential role in the sepal development and inflorescence determinacy (Vrebalov, et al. 2002), was sharply downregulated in both sepals and pistils in the *SIMBP22* overexpression plants compared to the WT. *TAG1* and *TAGL1*, two tomato C-class floral organ identity genes, are orthologs of *AGAMOUS* (*AG*) and *SHATTERPROOF1/2* (*SHP1/SHP2*) genes of Arabidopsis, respectively (Gimenez, et al. 2010; Pnueli, et al. 1994; Vrebalov, et al. 2009). In the overexpression transgenic plants, *TAG1* expression was dramatically upregulated in the pistils, but the *TAGL1* expression was greatly downregulated in both the stamens and pistils when compared with the WT (Fig. 2b, c). The transcript level of *SIMBP3*, one member of the class D MADS-box genes and specifies carpel/ovule identity, was evidently increased in the transgenic pistils compared to the wild-type (Fig. 2d). Two E-class genes, *TM5* and *TM29*, participate in the maintenance of floral meristem identity and the regulation of floral organ development (Ampomah-Dwamena, et al. 2002; Pnueli, et al. 1994). Relative to WT, the *TM5* showed increased expressions in the transgenic sepals, stamens and pistils (Fig. 2e). Fig. 2f showed that the transcript for the *TM29* was much lower in the transgenic pistils than that in the wild-type. Our results suggested that overexpression of *SIMBP22* leading to the morphological alterations of flowers might be attributed to the changes in the expressions of the floral organ identity genes.

Numerous researches have demonstrated that MADS-box transcription factors carry out their functions in flower development by forming dimers or higher-order complexes (Tonaco, et al. 2006). Subsequently, a yeast two-hybrid assay was performed to assess the self-activation of pGBKT7-*SIMBP22* and to confirm the interactions between the *SIMBP22* and other floral homeotic MADS-box proteins. As shown in Fig. 2g, no autoactivation activity was detected on SD/-Leu-Trp-Ade-His and SD/-Leu-Trp-Ade-His containing X- α -Gal plates. Besides, *SIMBP22* could physically interact with *MC*, *TM5* and *TM29* but not with *TAGL1*, *SIMBP3* in yeast. These results suggested that *SIMBP22* may carry out its role in flower development by forming dimers with *MC*, *TM5* and *TM29*, respectively.

Overexpression of *SIMBP22* results in reduced fecundity in tomato

The relatively high mRNA accumulation of *SIMBP22* in tomato fruit suggested the possibility of additional functions in fruit development (Supplementary Fig. S1) (Li, et al. 2020). Thus, the effects of the overexpression of the *SIMBP22* on fruit development were then investigated in the transgenic OE lines. We found another remarkable phenotype was that the strong overexpression lines (OE18 and OE2) could not bear fruit, whereas the mild overexpression transgenic lines (OE17, OE12 and OE14) showed reduced fruit size and produced fewer seeds. Occasionally, the strong overexpression line OE2 produced a much smaller fruit, while could not expand as normally as the wild type (Fig. 3a, b).

To further compare fruit development, some parameters were measured, including fruit weight, fruit volume, fruit diameter, pericarp thickness and the number of seeds in the B4 (4 d after breaker) stage fruits. As shown in Fig. 3c-e, the mild overexpression of *SIMBP22* resulted in significant reductions in fruit weight, fruit volume and fruit diameter when compared with those in the control fruits. Moreover, the pericarp thickness of fruits was also measured, and the result showed that the OE lines showed thinner pericarp tissues than WT plants (Fig. 3f). Additionally, compared to WT, the seed number per fruit of the mild overexpression transgenic plants were reduced by approximately 62% to 68% (Fig. 3g). Previous studies indicate that there are close relationships between fruit size and seed number per fruit (Hussain, et al. 2020), and then we speculate that the notable differences in fruit size between the mild *SIMBP22*-OE lines and WT plants might be attributed to significantly reduced pericarp thickness and seed yield.

Additionally, to further investigate whether the reduced fertility of transgenic flowers is a result of the defects in either the male or the female parts, cross-pollination experiments were conducted between wild-type and transgenic plants. The results showed that failed fertilization occurred by crosses between the wild-type pollen and strong transgenic pistils (data not shown), while seeds were produced successfully when mild overexpression transgenic flowers and wild-type flowers were respectively crossed with wild-type pollen and strong overexpression transgenic pollen (Fig. 3h). Besides, pollen viability was detected by TTC staining, and the result suggested that the strong overexpression transgenic pollen stained similarly to the WT pollen (Fig. 3i), hinting that the pollen viability may not be affected in the transgenic plants. Moreover, less viable pollen grains were observed in the strong overexpression transgenic flowers than in the WT flowers (data not shown). Subsequently, the relative transcript accumulation of pollen development-related genes *SICRK1* (Kim, et al. 2014), *SIPRALF* (Covey, et al. 2010), *LePRK3* (Kim, et al. 2002), *SIPMEI* (Kim, et al. 2013) were also examined by qRT-PCR assay. Intriguingly, all of these four gene transcripts were consistently reduced in the strong *SIMBP22*-overexpression lines compared to those in the non-transgenic plants (Fig. 3j-m), which were likely to be associated with the reduction of pollen grains in the transgenic lines with a strong *SIMBP22* overexpression. Thus, we propose that the infertility phenotype observed in the *SIMBP22*-OE transgenic tomato plants is probably attributed to low pollen production and defect in female reproductive development.

Overexpression of *SIMBP22* affects auxin signalling-related genes

Auxin plays critical roles in regulating fruit development, including fruit set and growth, ripening and abscission (Pattison, et al. 2014). Recently, we demonstrated that the mild overexpression of *SIMBP22* led to reduced plant height by affecting gibberellin (GA) and auxin homeostasis. (Li, et al. 2020). In this study, the artificial enhancement of *SIMBP22* resulted in infertility phenotype in the strong overexpression lines, and then we speculated that it was also related to the alteration of auxin signalling. Subsequently, qRT-PCR assay was conducted to further investigate the expression levels of auxin pathway-related genes in the wild-type and strong overexpression transgenic ovaries. It was found that the transcripts of an auxin biosynthesis gene *ToFZY5* (Exposito-Rodriguez, et al. 2011), an auxin response gene (*ARF3*) (Zouine, et al. 2014), three Aux/IAA transcription factor genes (*IAA13*, *IAA14* and *IAA29*) (Audran-Delalande, et al. 2012), were greatly increased in the *SIMBP22* strong overexpression transgenic ovaries at the anthesis stage, compared with the WT ovaries (Fig. 4a-e). By contrast, transcripts of an **AUX/LAX** gene *LAX1*, and three PIN genes (*PIN1*, *PIN2* and *PIN4*) (Pattison and Catala 2012), respectively encoding *auxin* influx and *efflux transport proteins*, were sharply decreased in the transgenic ovaries than in the WT (Fig. 4f, g and i). Relative to WT, *PIN2* gene expression was upregulated in the transgenic ovaries (Fig. 4h). Based on the results described above, we inferred that the overexpression of *SIMBP22* may alter tomato productive development via disturbing auxin signaling.

Overexpression of *SIMBP22* affects flowers and fruits size mainly by inhibiting cell expansion

Plant organ growth is controlled by multiple regulatory factors that coordinate cell proliferation and cell expansion (Anastasiou and Lenhard 2007; Horiguchi, et al. 2006). In our work, the *SIMBP22*-OE tomato plants exhibited reduced flower size, particularly first-whorl sepals, and smaller fruits with thinner fruit pericarp (Fig. 1c-e and Fig. 3b, f). Therefore, anatomical analyses were performed to investigate the cytological differences between the WT and transgenic sepals and fruit pericarps. Obviously, the cells in the sepal and pericarp respectively from the strong and mild *SIMBP22* overexpression transgenic lines were much smaller than those in the wild-type plants (Fig. 5a, b). Compared with the WT, the transgenic fruit pericarps contained slightly reduced cell layers, while the reduction in mean cell size of the mesocarp cell of the transgenic fruits reached up to a 60 % difference (Fig.5c, d). Furthermore, we analyzed the transcript levels of genes associated with plant cell division and cell expansion in the inflorescences from the WT as well as the strong *SIMBP22*-OE plants by qRT-PCR. *CDKA1*, involved in the progression of the cell cycle (Czerednik, et al. 2015; Czerednik, et al. 2012), showed no significant difference in the mRNA accumulation between the transgenic and non-transgenic plants (Fig. 6a). The transcripts for three cyclin genes, *SICycA3;1*, *SICycB1;1* and *SICycB2;1* were not clearly affected (Fig. 6b-d). In contrast, the expression levels of cell expansion-related genes, *SIEXP1* (Perini, et al. 2017), *LeEXP2* (Caderas, et al. 2000) and *LeEXP8* (Chen and Bradford 2000), were distinctly repressed in the *SIMBP22*-overexpressing plants (Fig. 6e-g). *FUL2*, as a member of the MADS-box transcription factor family, affects style abscission and cell expansion (Wang, et al. 2014). In the *SIMBP22*-OE plants, *FUL2* was strongly up-regulated when compared with its respective expression in the WT (Fig. 6h). Overall, these findings support the possibility that *SIMBP22* up-regulation leads to the alterations in tomato flowers and fruit size are, *atleastinpart*, due to the reduced cell expansion, rather than impaired cell division.

Mild overexpression of *SIMBP22* promotes proanthocyanidin accumulation and affects seed germination

The *tt16* seeds are yellow in color and the PA accumulation was restricted to the chalazal bulb and the micropylar end in the mutant seed coat, while the ectopic expression of *TT16* produced brown seeds as a result of ectopic PA biosynthesis (Nesi, et al. 2002). According to our observations, the *SIMBP22* overexpression transgenic seeds had a dark brown color (Fig. 7a). To further explain the phenotype regarding the pigmentation of the seed coat, a vanillin assay was conducted and indicated that the mild OE transgenic seeds may accumulate more PA than WT, and then we decided to measure the PA content. As expected, the OE plant seeds possessed higher PA levels than wild-type plants (Fig. 7b). The overaccumulation of pigments in the seed coat has a negative effect on seed germination (Debeaujon, et al. 2000). Subsequently, a seed germination assay was performed to try to detect the germination energy of the transgenic tomato seeds. The results exhibited lower seed germination rates in seeds from transgenic lines compared with those from the non-transgenic plants (Fig. 7c, d), implying that mild overexpression of *SIMBP22* may inhibit the germination ability of the transgenic tomato seeds.

Discussion

TT16/ABS/AGL32 gene has been identified as a member of the B_{sister} subfamily of MADS-domain proteins, which is well described in *Arabidopsis thaliana* (Kaufmann, et al. 2005; Mizzotti, et al. 2012; Nesi, et al. 2002; Xu, et al. 2017), *Petunia hybrida* (de Folter, et al. 2006; Tonaco, et al. 2006) and *Brassica napus* (Chen, et al. 2013; Deng, et al. 2012), respectively. In *Arabidopsis*, *TT16* is involved in the regulation of endothelium development and PA accumulation of developing seeds (Nesi, et al. 2002). The *FBP24*, is closely related to *TT16* in petunia, functions in endothelium layer development similar to previously described *TT16* in *Arabidopsis* (de Folter, et al. 2006). However, canola *TT16* ortholog *BnTT16* controls multiple physiological functions, especially seed oil synthesis and embryo development, which are beyond endothelial cell specification and flavonoid biosynthesis (Deng, et al. 2012). More recently, we have uncovered important biological roles of tomato *TT16* ortholog *SIMBP22* in plant vegetative development and drought stress responses (Li, et al. 2020). Furthermore, we found that the overexpressing *SIMBP22* transgenic tomato plants exhibited defects in *reproductive growth* and development, including morphological alterations of flowers, reduced fruit set and growth, and abnormal seed color. These data indicate that the functional conservation and diversity of *TT16* genes in different plant species.

B_{sister} genes are predominantly expressed in female reproductive organs, suggesting that this subfamily is involved in the evolution of the reproductive organs in seed plants (Becker, et al. 2002). Here, flowers of the *SIMBP22* overexpression plants showed considerable changes regarding floral organs size, *sepal and petal color* as compared with the wild-type, suggesting that the overexpression of *SIMBP22* in tomato may affect the development of flower, following what would be expected for a typical B_{sister} gene. To obtain further insights into the potential molecular regulation mechanism explaining the phenotypes associated with floral organ development, several MADS-box genes related to flower development were tested by qRT-PCR analysis in mature floral organs of both the WT and overexpression plants. These results

revealed that overexpression of *SIMBP22* caused alterations in the expression levels of these genes such as the close tomato of *SHP1/SHP2 (TAGL1)*, *SEP1/2 (TM29)*, *AG (TAG1)* and *SEP3 (TM5)* *AP1 (MC)* and *STK (SIMBP3)*. These results suggest that the impacts of *SIMBP22* on flower development may be associated with other transcription factors. It has been proposed that MADS-box transcription factors carry out their functions in floral organ formation and identity or other developmental processes by a complex network of protein-protein and protein-DNA interactions (Tonaco, et al. 2006). In the case of Arabidopsis, ABS/TT16 can form dimers with SEPALLATA (SEP) floral homeotic proteins and form higher-order complexes that also include the SEEDSTICK (STK) or SHATTERPROOF1/2 (SHP1, SHP2), which are verified by yeast-two-hybrid and three-hybrid assays, respectively (Kaufmann, et al. 2005). Hence, the formations of dimers and higher-order complexes may have a key role in regulating plant growth and development among MADS-box genes. In our study, a yeast two-hybrid experiment showed that *SIMBP22* could physically interact with a MADS-box transcription factor, MC, which is known as a regulator of sepal size (Vrebalov, et al. 2002), indicating that *SIMBP22* and MC can potentially form a dimer and then may explain the sepals with extremely reduced size observed in the *SIMBP22*-OE lines compared with WT. Meanwhile, protein-protein interactions were also observed in the yeast two-hybrid assay between *SIMBP22* and another two SEP MADS-box proteins, TM5 and TM29, which are previously verified to mediate organ differentiation of the inner three whorls of tomato flowers (Ampomah-Dwamena, et al. 2002; Pnueli, et al. 1994). It is possible that overexpressing *SIMBP22* can affect flower morphology at least partly be interpreted as the consequence of forming dimers, trimers, or even tetramers with other floral homeotic proteins in tomato plants.

The phases of fruit initiation and development have been considered to be the continuation of the floral developmental program, and MADS-box transcription factors play considerable and multiple functions during flower, fruit and seed development (Busi, et al. 2003). For instance, transgenic tomato plants with reduced *TAG1* expression levels exhibit defects in stamen and carpel identity, while overexpression lines display alteration in the first whorl, male and female sterility, and parthenocarpic fruit (Pnueli, et al. 1994). Another C class homeotic gene *TAGL1* plays an important role in both regulating fleshy fruit expansion and ripening processes. (Vrebalov, et al. 2009). The down-regulation of *SEP* homolog *TM29* leads to infertile stamens and ovaries, parthenocarpic fruit, and green-colored petals and stamens which suggest a partial conversion of these organs into sepals (Ampomah-Dwamena, et al. 2002) Besides modifying the flower morphology, we also found that the artificial enhancement of *SIMBP22* affected fruit set and growth, and the strong *SIMBP22* overexpression transgenic plants bore no fruit, while the smaller fruits with fewer seeds were observed in the mild overexpression transgenic lines compared to those in the WT. The strong *SIMBP22*-OE transgenic flowers were manually cross-pollinated with wild-type pollen, which failed to produce fruit, whereas normal seeds could develop when strong overexpression transgenic pollen was used to pollinate WT styles. In addition, TTC staining and qRT-PCR assays were carried out and the results showed that the pollen viability was not affected in *SIMBP22*-OE flowers, but the strong overexpression of *SIMBP22* led to reduced viable pollen grains. These data indicated that the strong overexpression of *SIMBP22* caused female sterility and disturbed mature pollen formation, thus resulting in reduced fertility.

The final size of an organ is controlled by two phases of growth, namely, cell proliferation and subsequent cell expansion (Anastasiou and Lenhard 2007; Horiguchi, et al. 2006). *SIMBP22* overexpressing plants showed smaller flowers and fruits with thinner pericarp and fewer seeds than those of the WT. qRT-PCR analysis was conducted and the results revealed that the expression of several genes involved in cell expansion, including *SIEXP1*, *LeEXP2* and *LeEXP8*, was greatly down-regulated in the OE plants, but the transcripts for cell cycle genes such as *CDKA1*, *SICycA3;1*, *SICycB1;1* and *SICycB2;1* displayed no obvious differences, when compared to their respective expression in the WT. These expression data agreed with the findings of the microscopic analysis, implying that the overexpression of *SIMBP22* affects flower and fruit size is likely to originate from the reduced cell expansion but not the impaired cell division. There have been reports highlighting the influence of phytohormones especially auxin and GA (de Jong, et al. 2009; de Jong, et al. 2011; Fuentes, et al. 2012; Hu, et al. 2018; Serrani, et al. 2008). Auxin response factor *SIARF7* may negatively regulate fruit set and moderates the auxin response during fruit growth (de Jong, et al. 2009). Silencing of *SI-IAA17* in tomato results in increased fruit size as a result of the alteration of endoreduplication-related cell expansion (Su, et al. 2014). Plants overexpressing transcription factor *SIGRAS24* affect fruit set and development via coordinating gibberellin and auxin homeostasis (Huang, et al. 2017). Taking into account two previous studies have shown that the transcript levels of *FBP24/ SIMADS29* (renamed here as *SIMBP22*) were greatly reduced after 2,4-D treatment in tomato ovaries by qRT-PCR analysis (Hu, et al. 2018; Tang, et al. 2015), indicate that *SIMBP22* may participate in the regulation of fruit development by mediating auxin signalling. Indeed, our qRT-PCR assay showed that the mRNA accumulations of several genes related to auxin signalling were altered in the transgenic ovaries. In addition, auxin signalling is also important to regulate floral organ size. For instance, tomato MADS-box gene *SIMBP21* negatively regulates cell expansion to affect sepal size, which was mediated by ethylene and auxin (Li, et al. 2017). Thus, it is likely that the overexpression of *SIMBP22* affected tomato reproductive development including floral organ size, fruit initiation and growth via disturbing auxin signalling, which is consistent with its effect on tomato plant vegetative development (Li, et al. 2020).

Proanthocyanidins (PAs), as one of the main flavonoids in Arabidopsis seeds (Routaboul, et al. 2012), play a crucial role in modulating seed dormancy and longevity during storage (Debeaujon, et al. 2000). Once oxidized, PAs confer brown pigmentation in the mature seed coat (Devic, et al. 1999). Arabidopsis *ttg1* mutant seeds have a yellow color, and appear reduced seed dormancy can be *ascertained by a higher germination rate* (Debeaujon, et al. 2000). Arabidopsis *TT16* gene participates in the control of seed coat pigmentation and proanthocyanidin (PA) accumulation in the endothelium of developing seeds (Deng, et al. 2012; Nesi, et al. 2002; Xu, et al. 2017). In our study, *SIMBP22*-OE transgenic seeds were dark brown in color, and *with more PA accumulation and* exhibited lower germination than non-transgenic seeds, indicating that the overexpression of *SIMBP22* in tomato not only affected biosynthesis of PAs but also altered seed dormancy. As reported, endogenous plant flavonoids not only affect seed germination and dormancy, but also play an important role in regulating cellular auxin efflux and consequent polar auxin transport. Auxin transport is elevated in *tt4* (no flavonoid production) but reduced in *tt7* and *tt3* mutants (which accumulate excess flavonols) (Peer, et al. 2004). Additionally, previous reports indicate

that seeds could produce or deliver auxins to the surrounding fruit tissues and then promote fruit expansion (Ariizumi, et al. 2013; Gillaspy, et al. 1993). The *agl62* mutant shows impaired transport of auxin was responsible for seed abortion (Figueiredo, et al. 2016). Our data demonstrated that overexpression of *SIMBP22* resulted in altered expression of a series of genes related to auxin transport. Based on the results described above, we infer that *SIMBP22* may act as a key regulator that affects flower development, seed *development, fruit set and growth via* affecting auxin signalling.

In summary, we *aimed to* investigate the effects of *SIMBP22* overexpression on the tomato reproductive development regarding flowers, fruits and seeds in this study. Our data demonstrate that the overexpression of *SIMBP22* not only results in morphological alterations of flowers but also affects fruit set, *fruit size and seed pigmentation. Morphological, physiological, and molecular analyses* have been carried out to preliminarily elucidate the causes related to the defects of *SIMBP22* overexpressing transgenic plants. These findings further advance our knowledge of physiological effects of B_s MADS-box transcription factors and provide valuable information for modern breeding biotechnologies.

Declarations

Author contributions Z.H., and G.C. designed research; F.L., Y.J., X.C., S.Z. and Q.X., performed the experiments; F.L. wrote the paper. All authors have read and approved the final version of the manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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Figures

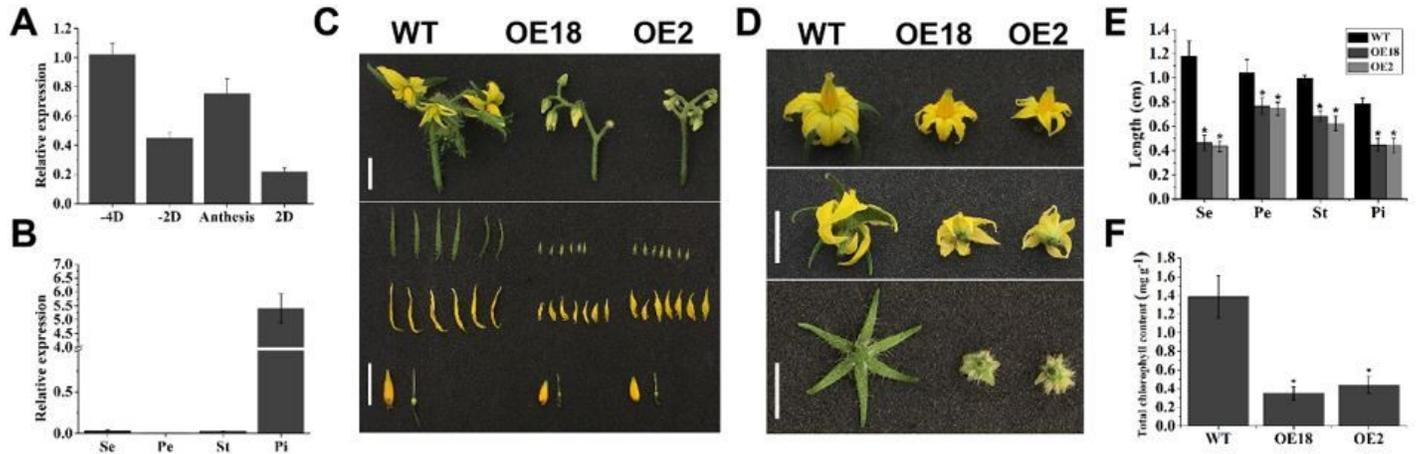


Figure 1

Overexpression of SIMBP22 alters tomato flower morphology. a Relative expression of SIMBP22 in flowers at different developmental stages. -4D, -2D, indicate flowers at 4d and 2d before anthesis; AD, anthesis day; 2D, flowers at 2d and post-anthesis. b Expression levels of SIMBP22 in different floral organs of the wild-type. Se, sepal; Pe, petal; St, stamen; Pi, pistil. c Inflorescence (upper) and four-whorl floral organs (bottom) from wild-type and SIMBP22-OE lines. OE18 and OE2, two independent SIMBP22-overexpressing lines. Bar =1 cm. d Flower (upper and middle) and sepal (bottom) phenotypes of the WT and transgenic plants. Bar =1 cm. e shows the maximum length of four types of floral organs from the wild-type and transgenic plants. Error bars indicate SE (n=11). f Total chlorophyll content in mature sepals. Asterisks indicate significant differences. (P < 0.05).

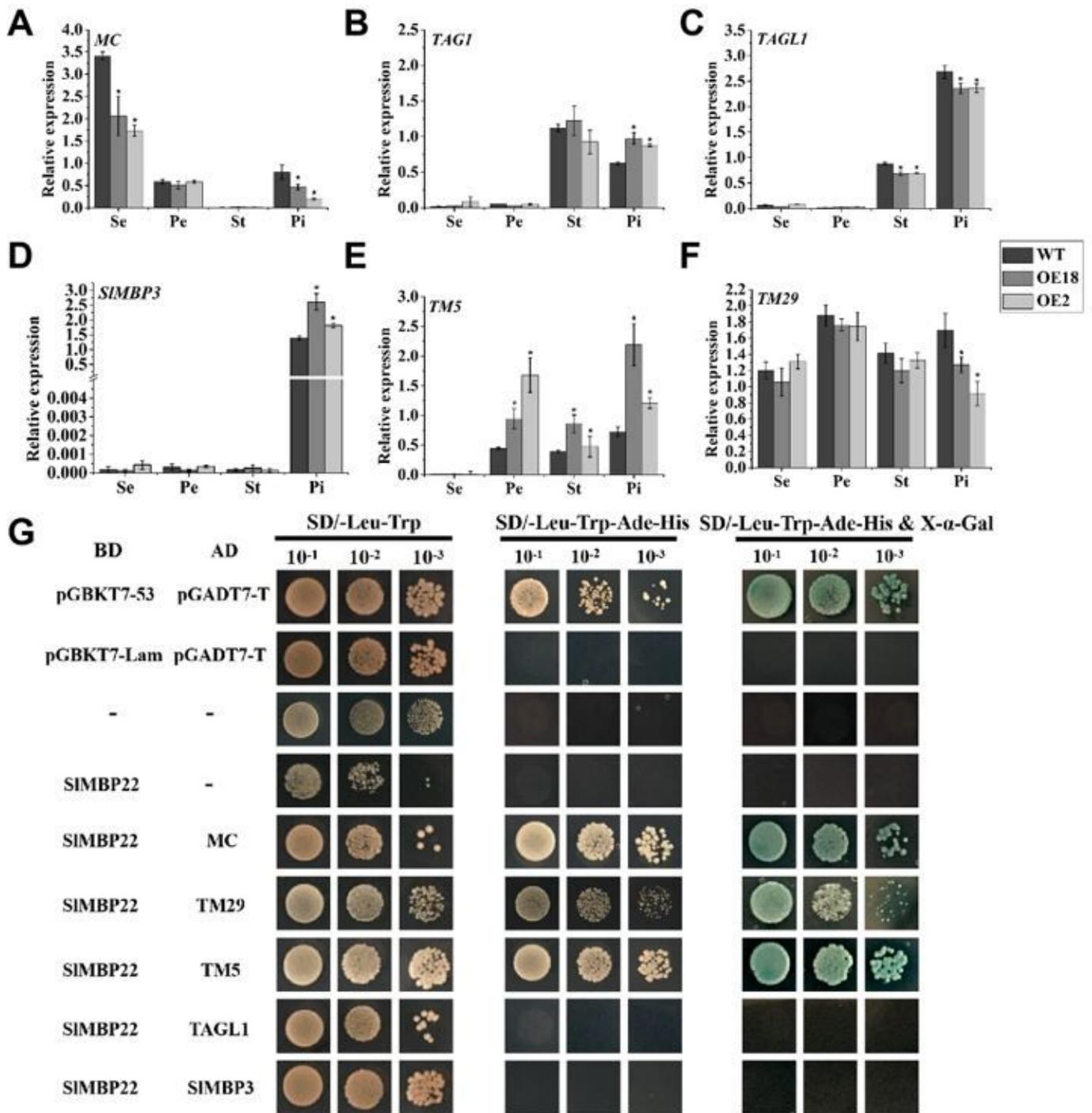


Figure 2

Overexpression of SIMBP22 affects floral organ identity genes. (a-f) respectively represents expression analysis of MC (A-class gene), TAG1 and TAGL1 (C-class genes), SIMBP3(D-class gene), TM5 and TM29 (E-class genes) in the wild-type and transgenic lines. Data are shown as the mean \pm SE of three biological replicates. Asterisks indicate significant differences compared with WT ($P < 0.05$). g Yeast two-hybrid assay for the SIMBP22 with MC, TAGL1, SIMBP3, TM5 and TM29. The protein interaction was detected on synthetic defined quadrupledropout (SD/-Leu-Trp-Ade-His) medium (middle) and which also

containing X- α -Gal (SD/-Leu-Trp-Ade-His & X- α -Gal) medium (right) after the yeast cells had been screened and had positive growth on SD double dropout (SD/-Leu-Trp) medium (left). pGADT7-T and pGBKT7-53, positive control. pGADT7-T and pGBKT7-Lam, negative control. The three columns (from left to right) correspond to three concentration gradients (10⁻¹, 10⁻², and 10⁻³). Combinations of empty bait and empty prey vector, and autoactivation assay with no yeast growth on SD/-Leu-Trp-Ade-His medium and SD/-Leu-Trp-Ade-His & X- α -Gal medium.

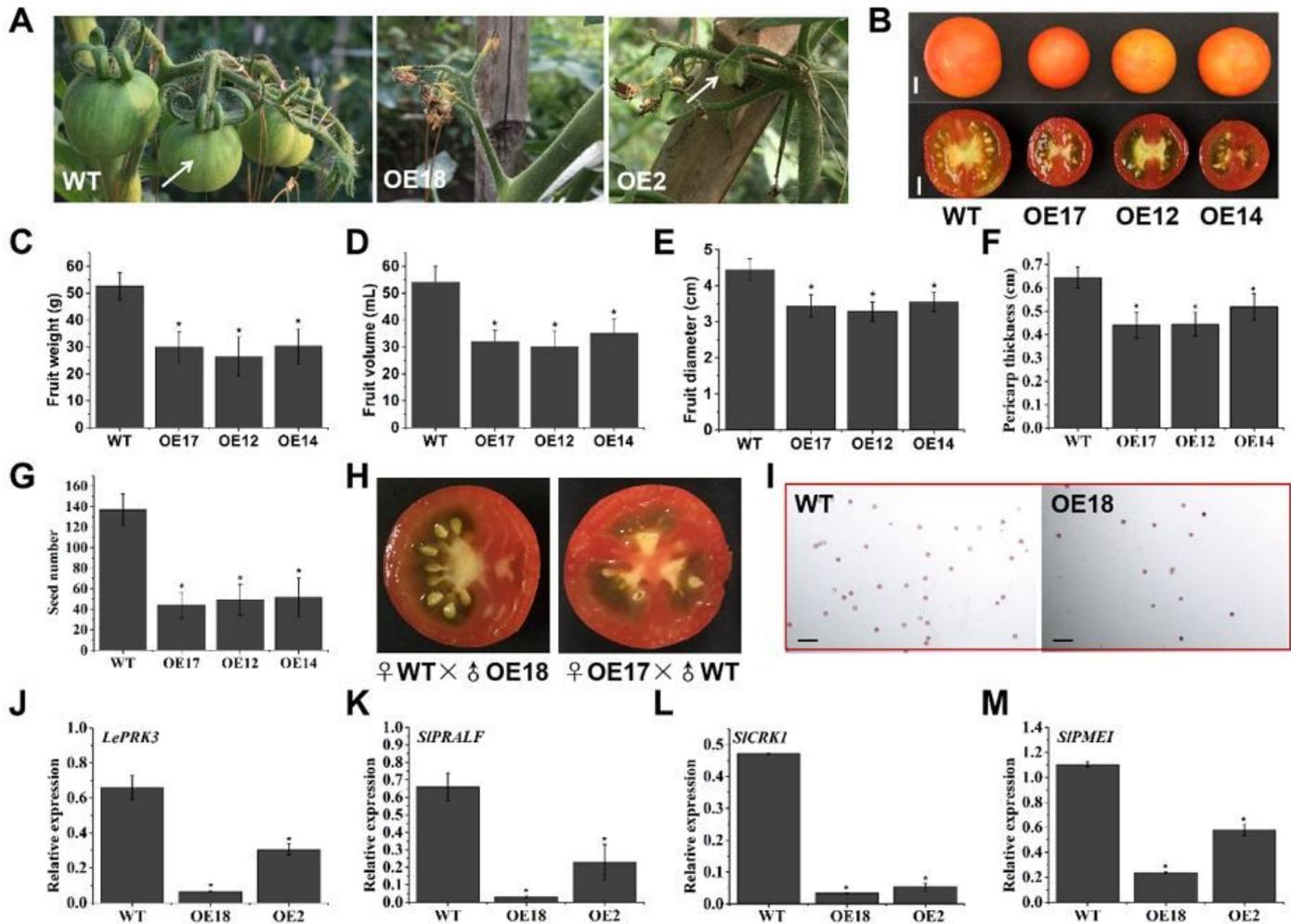


Figure 3

Overexpression of SIMBP22 results in reduced fecundity in tomato. a The Overexpression of SIMBP22 cause infertility phenotype observed in the strong transgenic lines, OE18 and OE2. The arrows indicate the OE2 plant occasionally produce a much small fruit which couldn't expand as normally as the wild fruits. b Representative fruits from the wild-type and mild overexpression transgenic lines, OE17, OE12 and OE14. Bar = 1 cm. Measurements of fruit weight (c), fruit volume (d), fruit diameter (e), pericarp thickness (f) and the number of seeds (g) in the B4 (4 d after breaker) stage fruits of mild transgenic OE plants. Each value represents the mean \pm SE of three biological replicates (n=15). (h) Fruits of the wild-type (left) and the mild overexpression transgenic line OE17 (right) produce seeds by manual crossing

assay. (i) Comparison of the pollen viability of WT and SIMBP22-OE line (OE18) with TTC staining. Bars = 50 μ m. The relative mRNA levels of pollen development-related genes LePRK3 (j), SIPRALF (k), SICRK1 (l) and SIPMEI (m) in the wild-type and transgenic plants. Error bars show the standard error between three biological replicates performed. Asterisks indicate significant differences compared with WT ($P < 0.05$).

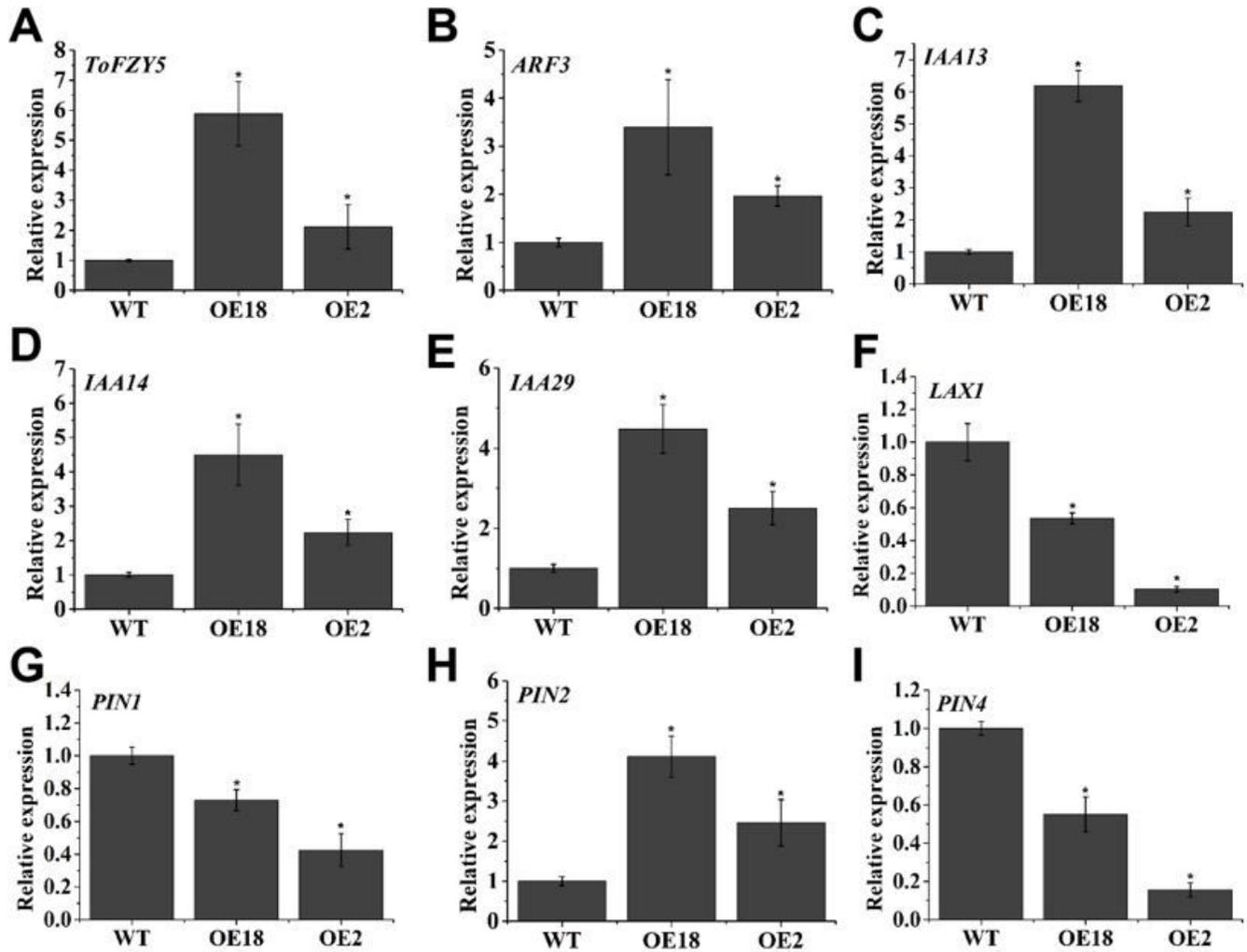


Figure 4

Overexpression of SIMBP22 affects auxin signalling-related genes. The relative expression levels of genes related to auxin biosynthesis (a), auxin response (b), Aux/IAA gene family (c-e), auxin influx (f) and efflux transport (g-i). Error bars indicate the standard error between three replicates performed. Asterisks indicate significant differences ($P < 0.05$).

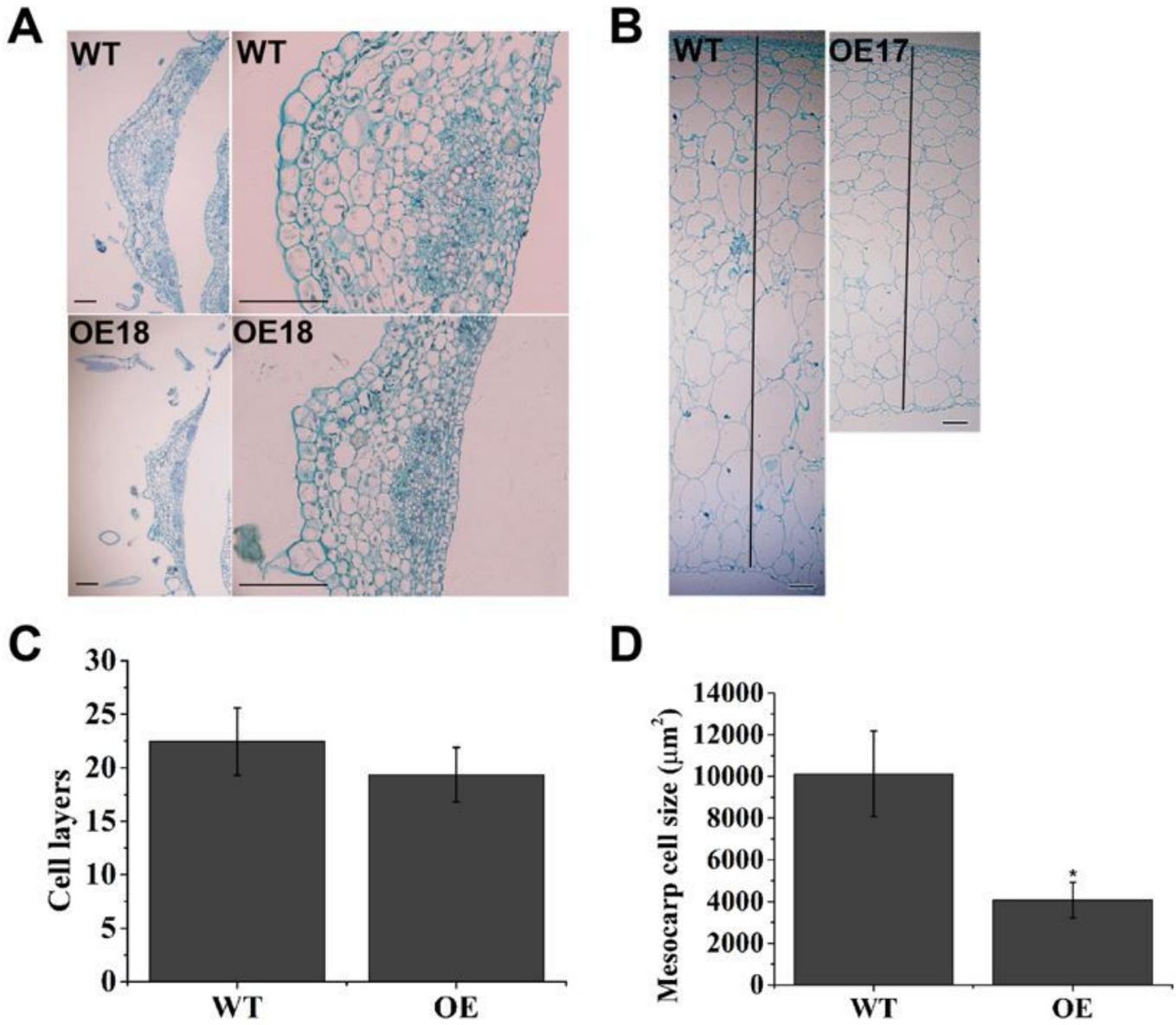


Figure 5

Anatomical analysis of sepals and fruits from the OE and WT plants. a Transverse section of sepals from the WT (upper) and SIMBP22-OE plants (bottom). Bars = 50 μm . b Transverse sections of fruit pericarp from the wild-type and transgenic fruits at breaker stage. The number of cells along black line was counted. Bars = 50 μm . The pericarp sections of the wild type (left) and OE (right) were counted for cell layers (c) and mesocarp cell size (d). Error bars are standard errors. The asterisks represent significant differences relative to wild-type tissues (($P < 0.05$ using Student's t-test).

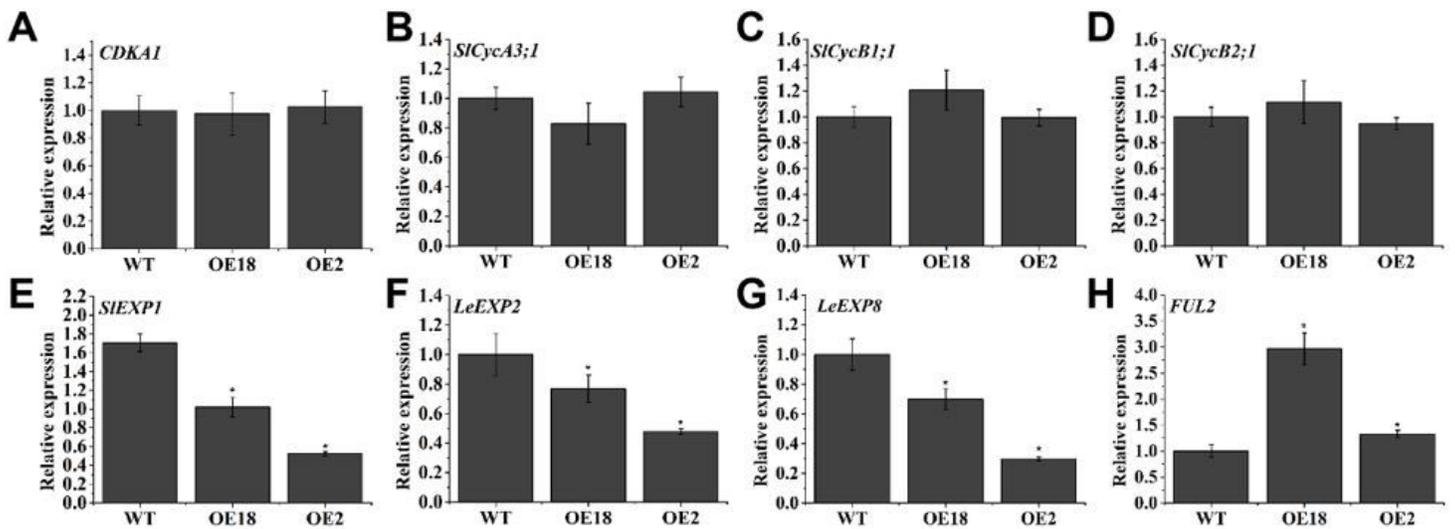


Figure 6

Expression levels analysis of genes related to cell cycle and cell expansion in wild-type and SIMBP22-OE transgenic plants. (a-d) show the transcription levels of *CDKA1*, *SICycA3;1*, *SICycB1;1* and *SICycB2;1* in the WT and transgenic plants, respectively. (e-h) Expression analysis of genes involved in cell expansion in the wild-type and transgenic plants. Each value represents the mean \pm SE of three biological replicates. Asterisks indicate significant differences between WT and OE plants ($P < 0.05$).

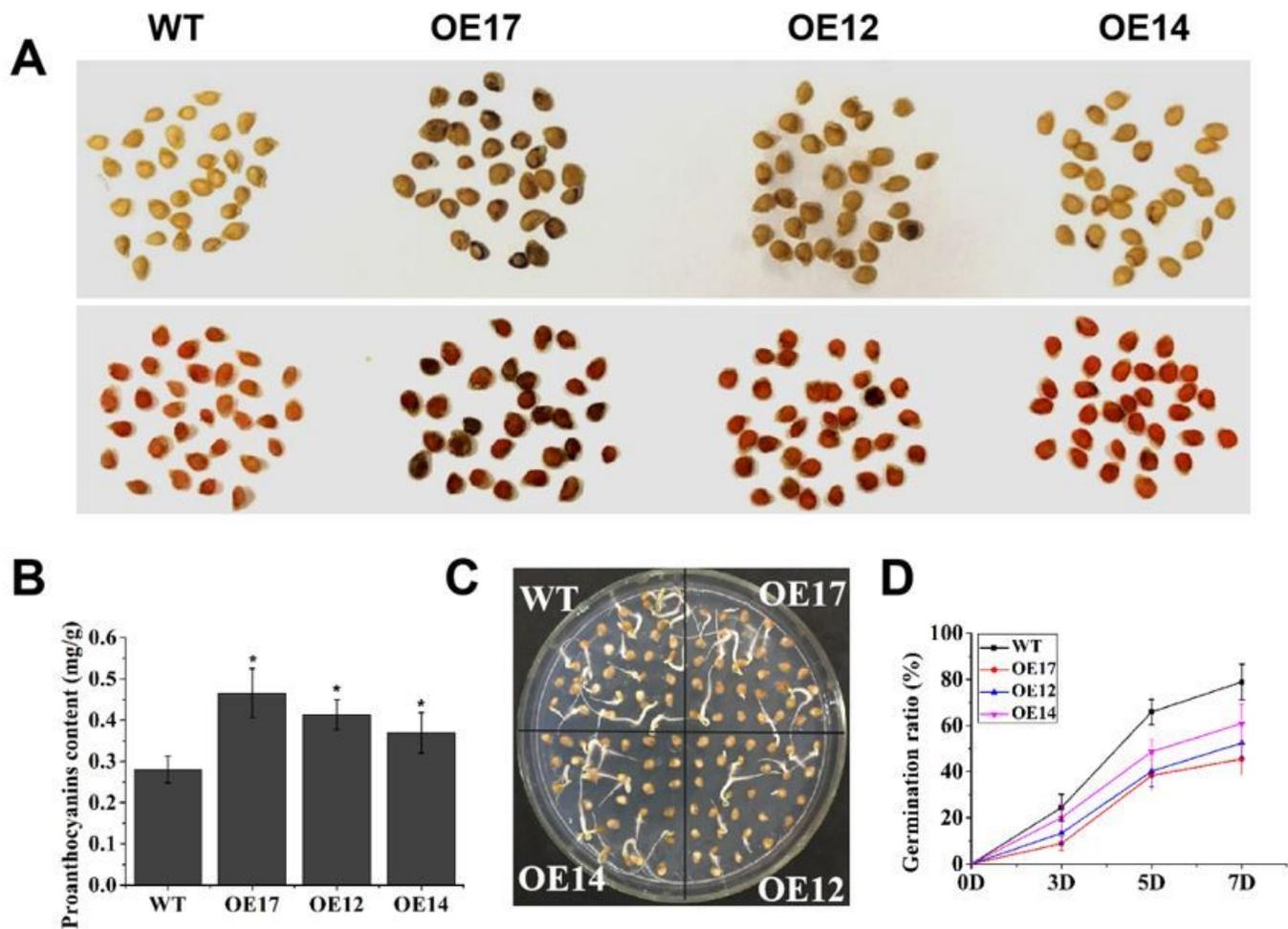


Figure 7

Phenotypes of the wild-type and SIMBP22-OE transgenic seeds. a A comparison of the seeds treated with vanillin reagent (bottom) and untreated seeds (upper). b Contents of proanthocyanidins of seeds from the wild-type and SIMBP22-OE plants. Results represent mean \pm SE from three biological replicates. Asterisks indicate statistically significant differences ($P < 0.05$). c Germination phenotype of WT and SIMBP22-OE transgenic seeds. d Germination rates of seeds on MS medium. Seed germination was scored on the third, fifth and seventh day after sowing. The data are the means \pm SE from three independent experiments with about 30 seeds per replicate.

Supplementary Files

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