Overexpression of SIOFP20 affects floral organ and pollen development

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Abstract
The OVATE gene was initially identified in tomato and serves as a key regulator of fruit shape. There are 31 OFP members in the tomato genome. However, their roles in tomato growth and reproductive development are largely unknown. Here, we cloned the OFP transcription factor SIOFP20. Tomato plants overexpressing SIOFP20 displayed several phenotypic defects, including an altered floral architecture and fruit shape and reduced male fertility. SIOFP20 overexpression altered the expression levels of some brassinosteroid (BR)-associated genes, implying that SIOFP20 may play a negative role in the BR response, similar to its ortholog OsOFP19 in rice. Moreover, the transcript accumulation of gibberellin (GA)-related genes was significantly affected in the transgenic lines. SIOFP20 may play an important role in the crosstalk between BR and GA. The pollen germination assay suggested that the pollen germination rate of SIOFP20-OE plants was distinctly lower than that of WT plants. In addition, the tomato pollen-associated genes SICRK1, SIPMEI, LePRK3, SIPRALF, and LAT52 were all suppressed in the transgenic lines. Our data imply that SIOFP20 may affect floral organ and pollen development by modulating BR and GA signaling in tomato.

Introduction
The reproductive development of most higher plants includes floral meristem determination, floral bud emergence, and fruit development and ripening, all of which result in seed formation and dispersal to guarantee offspring survival. Numerous studies indicate that several gene families extensively participate in the transcriptional modulation of reproductive and developmental processes. For example, the microRNA156-targeted SPL/SBP box transcription factors control the processes of ovary and fruit development in tomato. Silencing of SIDELLA, a GRAS transcription factor, induces facultative parthenocarpy in tomato fruits. A gain-of-function mutation in the MADS-box gene SI-AGL11 exerts a great impact on the organization of flowers and fruit, especially on the transition of the sepals into a carpel-like fleshy organ, and on increases in sugar content and fruit softness. The OVATE family proteins (OFPs) are plant-specific transcription factors. The OVATE gene was originally characterized as a key quantitative trait locus that contributes to the conversion of tomato fruit from round to pear shaped. Amino acid sequence analysis shows that this gene encodes a hydrophilic protein including a putative bipartite nuclear localization signal and a C-terminal domain of ~70 amino acids referred to as the OVATE domain. Unlike other known transcription factor families, the OVATE proteins are a novel class of functional proteins. OFPs have mainly been functionally characterized in Arabidopsis and rice and have been demonstrated to control diverse aspects of plant growth and development. Overexpression of AtOFP1 decreases the length of all aboveground organs, such as the hypocotyl, rosette leaf, floral organs and siliques, and chromatin immunoprecipitation analysis demonstrated that AtOFP1 directly regulates AtGA20ox1, encoding the key enzyme in GA biosynthesis. Additionally, AtOFP1 may take part in DNA repair. Moreover, plants overexpressing AtOFP2, 4 and 7 generate similar phenotypes to AtOFP1.
overexpressing plants, such as kidney-shaped cotyledons and round and curled leaves\(^7\),\(^10\). Overexpression of OsOFP2 results in decreased plant height and an altered leaf morphology and seed shape in rice\(^12\). Overexpression of OsOFP1 in rice causes multiple phenotypes, including increased leaf angles, decreased plant height, and altered grain shape\(^14\). The interaction of MaOFP1 and MuMADS1 in banana plays an antagonistic role in ethylene-induced postharvest fruit ripening\(^16\). According to the most recent study, MuMADS1 and MaOFP1 control fruit quality in a tomato ovate mutant\(^17\). These results strongly support the notion that OFPs act as important regulatory factors in numerous processes in plant growth and development.

Studies on the mechanism of action of OFPs have shown that they function via interacting with different kinds of transcription factors, such as KNOX and BELL classes\(^5\),\(^13\),\(^18\). The interaction networks between OFPs and TALE proteins have an important effect on plant developmental processes. The interaction of AtOFP1 and BLH3 has been shown to regulate the timing of conversion from the vegetative to reproductive stage in Arabidopsis\(^11\). AtOFP4 has been proposed to interact with KNAT7 (Knotted1-Like Homeodomain Protein 7) to control the establishment of secondary cell walls by increasing the transcriptional repression activity of KNAT7\(^7\),\(^10\). The interaction of AtOFP5 with KNAT3 and BLH1 inhibits the activity of BELL–KNOX TALE complexes to assure normal embryo sac development in Arabidopsis\(^19\). In Arabidopsis, the cell wall defect of the knat7 mutant can be partially restored by ectopic expression of GhKNL1, a homeodomain protein from cotton (Gossypium hirsutum); moreover, GhKNL1 can interact with GhOFP4, AtOFP1, and AtOFP4\(^20\).

Hormone pathways extensively participate in the extraordinary plasticity of plant ontogeny. There are several classes of phytohormones, including auxins, brassinosteroids, and gibberellins, that play essential roles in the regulation of growth in general and of cell elongation in particular\(^24\). Previous reports suggest that OFPs affect plant developmental processes by modulating the brassinosteroid and gibberellin signaling pathways\(^7\),\(^13\),\(^15\). AtOFP1 controls cell elongation in part by regulating the mRNA accumulation of the gibberellin biosynthesis gene AtGA20ox\(^1\). OsOFP19, OSH1 and DLT form a complex that regulates the complicated balance between plant growth and development and brassinosteroid signaling\(^7\). Increased brassinosteroid signaling can induce the expression of OsOFP1 by OsBZR1 and promote protein stability by repressing OsGSK2, resulting in the activation of OsOFP1, which then bonds with DLT factors and regulates downstream genes such as gibberellin metabolism genes to control plant morphology and grain shape in rice\(^14\). OsOFP8 acts as a positive regulator in the brassinosteroid signaling pathway by interacting with OsGKS2, which plays a negative role in the brassinosteroid signaling pathway\(^13\).

In view of the outstanding nutritive and commercial value of tomato (Solanum lycopersicum), it has been regarded as one of the most important vegetable crops. It is also a model organism for studying fleshy fruit development and ripening\(^25\), compound leaf development, and floral system and plant architecture\(^22\). Genome-wide analysis of OFPs in tomato has been carried out, and there are 31 SIOFPs in the tomato genome\(^24\). Herein, we present the functional characterization of SIOFP20 (accession number: Solyc10g076180), a classic OFP family gene homologous to AtOFP1 and OsOFP19 in Arabidopsis and rice, respectively. It has been reported that SIOFP20 is a suppressor of ovate in the modulation of fruit shape\(^25\). To investigate the role of SIOFP20 related to the development of vegetative and reproductive growth in tomato, the SIOFP20 gene was cloned and overexpressed in wild-type tomato, leading to pleiotropic phenotypes. In this study, we sought to reveal the impacts of SIOFP20 on reproductive development, including floral architecture and pollen development. Morphological, statistical, and molecular evidence is reported here to clarify the potential reasons for these phenotypes.

### Materials and methods

#### Plant materials and growth conditions

Solanum lycopersicum Mill. cv. Ailsa Craig tomato plants were used as the wild-type (WT) in our research. WT and transgenic tomato plants were grown in a greenhouse under standard greenhouse conditions (16-h-day/8-h-night cycle, 25 °C/18 °C day/night temperature). To determine the organ-specific expression pattern of SIOFP20, roots, stems, leaves, sepals, flowers, and fruits of different stages were sampled from WT tomato plants according to our previous report\(^26\). The four-whorl floral organs (sepal, petal, stamen, and carpel) were also harvested. For 24-epibrassinolide (EBR) treatment, 10 μM EBR and water (control) were sprayed on five-leaf-stage wild-type tomato plants. In addition, the third leaves from treated and untreated plants were harvested after 0, 1, 2, 4, 8, 12, and 24 h. All samples used in this study were immediately frozen with liquid nitrogen and kept at −80 °C.

#### Sequence analysis and phylogenetic tree construction

The protein sequence alignment of SIOFP20 and other OFP proteins was generated by using the DNAMAN 5.2.2 programs. The conserved OVATE domains were identified by using Scan Prosite (http://prosite.expasy.org/scanprosite/) to reveal the phylogenetic relationships of SIOFP20 with 17 OFP family proteins from Arabidopsis and rice. The maximum likelihood (ML) method was applied to construct a dendrogram with MEGA 6.06 software. The accuracy of this tree was ensured by the bootstrap test replicated 1000 times.
The GenBank accession numbers of the proteins included in the tree were as follows: AtOFP1 (NP_195804), AtOFP2
(NP_180599), AtOFP4 (NP_172174), AtOFP5 (NP_193618), AtOFP6 (NP_680125), AtOFP7 (NP_179440), AtOFP8
(NP_197466), AtOFP13 (NM_196102), AtOFP15 (XM_565833), AtOFP16 (NP_180770), AtOFP18
(NP_566967), OsOFP1 (XP_015638684), and OsOFP19
(XP_015638848). The Tomato Solanacea Genomics Net-
work (SGN) unigene accession numbers were as follows:
SlOFP5 (Solyc02g072030), SlOFP14 (Solyc06g082460),
SlOFP15 (Solyc07g055240), SlOFP17 (Solyc09g018200),
and SlOFP20 (Solyc10g076180).

Vector construction and tomato transformation
For the overexpression of SlOFP20 in WT tomato, the full-
length sequence was amplified by high-fidelity PCR
(Prime START mix DNA polymerase, Takara) with the
SlOFP20-F and SlOFP20-R primers (Supplementary Table
S1), which were tailed with XhoI and SacI restriction sites,
respectively, at their 5’ ends. A DNA-Tailing kit (Takara)
was applied to tail the obtained PCR products, which were
then linked with the pMD18-T vector (Takara). The correct
pMD18-T-SlOFP20 plasmid was used as the template and
was amplified with the primers SlOFP20-F and SlOFP20-R.
Then, the amplified products were inserted into the pBI121
vector. The resulting SlOFP20-OE vector was introduced
into Agrobacterium LBA4404. Plant transformation was
conducted following our previously published protocols.
Transgenic lines were screened on kanamycin medium and verified by genomic PCR using the
NPTII-F and NPTII-R primers (Supplementary Table S1). The positive SlOFP20-OE transgenic lines were retained and used for further studies.

Gene expression analysis
In this study, RNAiso Plus (Takara) was used to extract
total RNA. One microgram of total RNA was reverse-
transcribed (M-MLV Reverse Transcriptase Kit, Promega).
Transcript levels were evaluated by real-time quantitative PCR according to a method published previously. SlCAC (Solyc08g006960) was used as a reference
gene. The primer sequences are shown in Supplementary Table S1.

Transactivation activity and yeast two-hybrid assay for SlOFP20
Transactivation activity and yeast two-hybrid assays were performed according to our previous report.

Anatomic characterization and scanning electron microscopy
For anatomic characterization, WT and OE3 transgenic lines
were fixed with 70% ethanol/acetic acid/formaldehyde (18:1:1, v/v/v). Paraffin sections were prepared
for SEM (scanning electron microscopy) analysis, fully
open flowers from the WT and OE3 transgenic lines were collected and transferred to germination solution. Released pollen was then germinated by incubation in the dark at 25 °C for 3 h and were defined as germinated when the pollen tube was at least as long as the diameter of the pollen grain. Pollen germination was observed, and images were taken. A pollen viability assay was conducted as described previously. Pollen grains of WT and OE3 plants at the anthesis stage were collected and soaked in a 0.1% 2,3,5-triphenyl-2-h-tetrazolium chloride (TTC) solution for 15 min to assess their activity. The experiments were repeated three times.

Cross assay
A cross assay was performed according to our previous report. In brief, unopened flower buds from OE3 transgenic lines were emasculated. Mature pollen from the WT was transferred by brushing the WT anthers onto the stigmas of the OE3 transgenic lines.

Seed germination assay
Seeds from WT and SlOFP20-OE transgenic tomato
lines (T2) were used for germination assays. After surface sterilization, seeds (~30 seeds for each replicate) were sown onto MS medium and then germinated in the dark at 25 °C for 10 days. Radicle emergence >1 mm was regarded as seed germination. Seed germination rates were recorded daily. The experiments were repeated three times.

Results
SlOFP20 is a typical OVATE family protein
OVATE family proteins govern various developmental processes. To study the potential roles of OFP genes in tomato, we isolated an OFP gene (SlOFP20) from WT tomato on the basis of the sequence available in GenBank
(accession no. XM_004248997). The SlOFP20 gene is intronless and has an open reading frame (ORF) of 966 nucleotides, which encodes a protein of 321 amino acid residues. Multiple sequence alignment analysis of SlOFP20 and other well-known OVATE family proteins
from *Arabidopsis* and rice indicated that the SlOFP20 protein possesses a typical OVATE domain in the C-terminus (Fig. 1a). Phylogenetic analysis was carried out to study the relationship between tomato SlOFP20 and members of the *Arabidopsis* and rice OVATE family proteins (Fig. 1b), revealing that this tomato protein can be classified into a distinct clade that includes AtOFP1 and OsOFP19, its putative orthologues from *Arabidopsis* and rice, respectively.

A yeast two-hybrid system was applied to study the transcriptional activity of SlOFP20. A GAL4 DNA-binding domain SlOFP20 fusion protein was expressed in Y2H yeast cells to evaluate their capacity to initiate transcription from the GAL4 sequence. SlOFP20 could not promote yeast growth in the absence of histidine and adenine (Fig. S1), indicating that SlOFP20 does not exhibit transactivation activity.

**Expression patterns of SlOFP20**

To predict the potential function of SlOFP20 underlying tomato growth and development, quantitative reverse transcription-PCR (RT-qPCR) was applied to examine its expression patterns in various tomato organs. As shown in Fig. 2a, the results suggested that SlOFP20 showed the highest transcript accumulation in the roots, followed by the stems and flowers, while relatively low transcript levels were present in the leaves and IMG fruits. SlOFP20 mRNA was not detected in MG, B, B+4, and B+7 fruits. In addition, the expression level of SlOFP20 in the four-whorl flower organs in WT tomato was analyzed, indicating that SlOFP20 was mainly present in the sepal s, stamens, and carpels (Fig. 2b). These results suggested that SlOFP20 showed tissue-specific expression in tomato and may be involved in the development of roots, stems, and flowers.

**Overexpression of SlOFP20 alters tomato flower and fruit morphology**

Previous studies on OFP members in *Arabidopsis* revealed that single or multiple knockout mutants of OFP members do not display morphological defects. In contrast, overexpression of some OFP members generates obvious morphological alterations, indicating that these family members have redundant functions. There are 31 OFP family members in tomato, and SlOFP20 is closely related to SlOFP14. In addition, the Atofp1-1 mutant, in which the putative orthologous gene of SlOFP20 in *Arabidopsis* is mutated, does not show evident morphological changes. Downregulation of SlOFP20 in wild-type tomato does not impact fruit shape. Thus, we inferred that SlOFP20 may exhibit functional redundancy with other OFP members in tomato. For this reason, we overexpressed SlOFP20 in wild-type tomato to investigate its possible functions in tomato growth and development. The full-length SlOFP20 gene fragment was cloned into a plant overexpression vector (pBI121), which was then transferred to WT tomato. Nine independent transgenic lines were obtained. The mRNA accumulation of SlOFP20 was upregulated in the leaves of all transgenic lines. The 35S:SlOFP20 plants exhibited numerous morphological defects related to vegetative and reproductive organs, indicating a prominent role of SlOFP20 in a wide range of tomato growth and developmental phases. One of the most distinct alterations was plant sterility, observed in the strong overexpression transgenic lines. Thus, we selected the mild overexpression transgenic lines OE3,
OE7, and OE8, which produced seeds, for further investigation. The representative overexpression efficiency of the T1 generation of OE3, OE7, and OE8 transgenic lines was evaluated by RT-qPCR (Fig. 2c). We also identified strong overexpression plants in the T1 generation transgenic lines, which showed a number of abnormal phenotypes, including plant growth retardation, exserted stigmas, and an altered vegetative and floral architecture.

In this study, we focused on these phenotypes associated with reproductive development. Compared to WT, the flowers of OE plants were shorter, but the floral organs, including the sepals, petals, and stamens, were wider (Fig. 3a–c). Therefore, the length and width of the sepals, petals, and stamens in WT and OE3 plants were measured (Fig. 3g, h). The results showed that the lengths of the sepals, petals, and stamens were significantly reduced in OE3 plants, while the widths were greater than in WT. The shape indexes (length/width) of the sepals, petals, and stamens was also calculated, which showed a remarkable reduction in OE3 plants (Fig. 3i). As shown in Fig. 3c, the flowers of OE line plants showing strong overexpression exhibited exserted stigmas, which prevented pollination and resulted in sterility; thus, we could not observe a phenotype related to tomato fruit. However, the mild overexpression plants in the T1 generation transgenic lines could produce fruits, and the changes in fruit shape resembled those in the flowers (Fig. 3e, f).

To determine the cytological difference between WT and SIOFP20-OE flowers, anatomical analysis of the flowers at anthesis was conducted. Compared to WT, the transverse sections of OE3 sepals, petals, and stamens were much thicker due to an increase in the cell layer number and a larger cell size (Fig. 4).

**Overexpression of SIOFP20 in tomato affects BR- and GA-related genes**

BR and GA are two principal phytohormones that function redundantly in promoting plant growth. The most visible phenotype of BR- and GA-deficient plants is dwarfed growth. Here, the phenotypes caused by overexpression of SIOFP20 in tomato strongly resembled those of plants lacking BR and GA. Overexpression of AtOFP1 in Arabidopsis results in reduced cell elongation, which has been partially attributed to the suppression of gibberellin biosynthesis⁷. Moreover, a recent study showed that OsOFP19 negatively regulates the brassinosteroid (BR) response in rice¹⁵. EBR treatment continuously inhibited the expression of SIOFP20 (Fig. 5a).
Therefore, we speculated that overexpression of \textit{SlOFP20} inhibited plant growth by controlling the BR and GA pathways. To verify this speculation, the relative expression levels of some BR- and GA-related genes were detected by RT-qPCR. In rice, OsOFP19 increases the transcriptional activity of \textit{OSH1} but suppresses \textit{DLT15}. The homologous genes of \textit{OSH1} and \textit{DLT} in tomato are \textit{KONX1} and \textit{GRAS41}, respectively. Similarly, the transcript level of \textit{KNOX1} in \textit{SlOFP20}-OE transgenic lines was sharply increased (Fig. 5b), whereas the expression of \textit{GRAS41} was significantly inhibited compared to that in WT plants (Fig. 5c).

The expression profile of \textit{KNOX1} obtained from the Tomato eFP Browser (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi) showed that KNOX1 was mainly expressed in roots and flowers (Fig. S2). The \textit{GRAS41} transcript also primarily occurred in roots and flowers\textsuperscript{34}. \textit{SlOFP20}, \textit{KNOX1}, and \textit{GRAS41} showed similar expression patterns in tomato, implying that they may function together to regulate plant growth and development. To confirm this possibility, a yeast two-hybrid assay was used to examine their interactions. The results suggested that \textit{SlOFP20} could interact with \textit{KNOX1} and \textit{GRAS41}, and \textit{KNOX1} and \textit{GRAS41} showed a definite interaction (Fig. 5i). In addition, we assessed the mRNA abundance of the BR biosynthesis genes \textit{CPD}, \textit{D2} (an ortholog gene of rice \textit{OsD2}), and \textit{DWARF} in \textit{SlOFP20}-OE transgenic lines, and the results indicated that the levels of these BR biosynthesis genes were remarkably increased in \textit{SlOFP20}-OE plants (Fig. 5d–f). Moreover, the BR catabolism gene \textit{CYP734A7} was increased in \textit{SlOFP20}-OE plants (Fig. 5g).

The upregulation of BR biosynthesis genes may be due to feedback regulation, and the BR receptor \textit{BRI1} is essential for the homeostasis of endogenous BR contents\textsuperscript{35}. Thus, the expression level of \textit{BRI1} was measured and was shown to be significantly increased in \textit{SlOFP20}-OE transgenic lines (Fig. 5h). Similar results were found in \textit{OsOFP19}-OE transgenic rice plants\textsuperscript{15}. Based on these results, we inferred that the mechanism whereby \textit{SlOFP20} regulates BR signaling may closely resemble that of its orthologue \textit{OsOFP19} from rice.

On the other hand, \textit{AtOFP1} reduces the mRNA accumulation of the GA biosynthesis gene \textit{AtGA20ox1} via binding to \textit{KNAT1}, corresponding to \textit{KNOX1} in tomato\textsuperscript{6,7}. Therefore, we also evaluated the transcript accumulation of GA biosynthesis genes in wild-type and \textit{SlOFP20}-OE plants. Three genes involved in the early steps of GA biosynthesis, \textit{CPS}, \textit{KS}, and \textit{KAO}, were...
markedly increased in SlOFP20-OE transgenic plants (Fig. 6a–c). GA20oxs are also major GA biosynthetic enzymes, and GA3oxs catalyze the final step in the generation of bioactive GAs (GA1, GA3, and GA4) \(^{36-38}\). The expression levels of GA20ox1, GA3ox1, and GA3ox2 were dramatically increased in SlOFP20-OE plants compared with wild-type plants (Fig. 6d–f). The expression levels of GA2ox1 and GA2ox2, which encode GA2oxs (the main GA catabolic enzymes) \(^{39}\), were also detected, and the data suggested that the levels of both GA2ox1 and GA2ox2 were sharply increased in SlOFP20-OE plants (Fig. 6g, h).

Generally, the length of plant organs is determined by cell number and cell length, which are associated with cell division and cell elongation, respectively. There are hundreds of target genes downstream of the BR and GA pathways, including cell division and cell elongation genes; thus, we attempted to detect the transcript levels of some genes associated with cell division and cell elongation by RT-qPCR. The cell cycle regulatory gene CDKA1 was distinctly downregulated in SlOFP20-OE transgenic lines (Fig. 7a). The transcription accumulation of four cyclin genes was checked. SlCycA3;1, SlCycB2, and SlCycD2;1 were notably repressed in SlOFP20-OE transgenic lines (Fig. 7b, d, e), but SlCycB1;1 (Fig. 7c) was not affected. We also detected the mRNA transcript levels of E2FA and SlCYCT1;3 (Fig. 7f, g), two cell cycle-associated genes that participate in the G1 to S transition, but no distinct changes were found between the WT and SlOFP20-OE transgenic lines. The PRE (Paclobutrazol resistance) family of small helix-loop-helix (HLH) proteins positively regulates plant cell elongation \(^{40,41}\). There are five putative PREs (PRE1-5) in tomato, and the expression levels of these five genes were examined (Fig. 8a–e); compared to WT, all of them were notably suppressed in the SlOFP20-OE transgenic lines.

**Overexpression of SlOFP20 reduces male fertility**

In this study, we found that the strong SlOFP20-OE tomato plants could not bear fruit, which may be due to the exerted stigmas of SlOFP20-OE tomato flowers. We wondered whether the development and function of the pollen in SlOFP20-OE tomato flowers was also affected. The quality of pollen is crucially important in the reproductive stage of most plant species. Hence, a pollen germination experiment was carried out to evaluate the impact of SlOFP20 overexpression on pollen vitality. The results suggested that the pollen germination rate of transgenic plants was distinctly lower than that of WT plants (Fig. 9a, b). Further statistical analysis indicated that the pollen germination rate of SlOFP20-OE plants
Fig. 5 Overexpression of SlOFP20 affects BR-related genes. a The expression levels of SlOFP20 at different time points under 10 μM EBR treatment. b–h Comparison of BR-related gene expression between WT and overexpression lines. Each value represents the mean ± SE of three replicates. * indicates a significant difference (P < 0.05) between the wild-type and transgenic lines.

Yeast two-hybrid assay for the SlOFP20 & SlKNOX1, SlOFP20 & SlGRAS41 and SlKNOX1 & SlGRAS41 proteins. QDO, SD medium without Trp, Leu, His, and Ade; QDO/X, QDO medium with X-a-Gal. 1. pGBK7-53 & pGADT7-T (positive control); 2. pGBK7-Lam & pGADT7-T (negative control); 3. pGBK7-SlOFP20 & pGADT7-SlKNOX1; 4. pGBK7-SlOFP20 & pGADT7-SlGRAS41; 5. pGBK7-SlKNOX1 & pGADT7-SlGRAS41; Empty bait vector, empty prey vector, and autoactivation assay with no growth of yeast.

Fig. 6 Overexpression of SlOFP20 affects GA-related genes. Comparison of GA-related gene expression between WT and overexpression lines (a–g). Each value represents the mean ± SE of three replicates. * indicates a significant difference (P < 0.05) between the wild-type and transgenic lines.
was 2.36-fold lower than that of WT plants (Fig. 9e). Moreover, TTC staining for pollen viability suggested that there were fewer pollen viable grains in SlOFP20-OE flowers than in WT flowers. These results suggested that overexpression of SlOFP20 may impair male fertility. When the flowers of SlOFP20-OE plants were manually crossed with WT pollen, normal seeds could develop, hinting that the female fertility of SlOFP20-OE transgenic plants may not be affected (Fig. 9f).

A scanning electron microscope was employed to observe the stamen morphologies of WT and transgenic plants. The stamen epidermal cells of the transgenic plants were shorter and more intense than those of WT, and the cell arrangement in OE3 had a scale-like appearance (Fig. 10a, b). The shape of the pollen grains in WT and the OE3 transgenic plants did not show obvious changes (Fig. 10c, d). The grains of both plant lines presented a normal globular shape. In addition, we detected three floral organ identity genes, TAG1, TAGL2, and TM5. TAG1 is a C-class gene that has been suggested to take part in the specification of stamen and carpel identities. TM5 and TAGL2 (syn. TM29) are E-class genes. The expression levels of TAG1
and TAGL2 were not obviously altered in SIOFP20-OE transgenic lines (Fig. 10e, g), while that of TM5 was evidently increased in SIOFP20-OE transgenic plants (Fig. 10f). Moreover, the expression levels of pollen development-specific genes were evaluated in WT and SIOFP20-OE tomato plants. SICRK1, a cysteine-rich receptor-like kinase, plays a critical role in pathogen protection and programmed cell death. Pectin methylesterase inhibitor (SlPMEI) acts as a key regulator of pectin methylesterase (PME). LePRK3, a pollen-specific receptor kinase gene, may take part in perceiving extracellular cues during pollen tube growth. SIPRALF, an exogenous rapid alkalization factor, negatively adjusts the elongation of the pollen tube, and LAT52 may participate in germination or early tube growth. The results showed that all five of these genes were remarkably repressed in SIOFP20-OE lines, indicating that the overexpression of SIOFP20 suppressed the mRNA expression of these genes, resulting in reduced pollen fertility (Fig. 11a–e).

Mounting evidence suggests that numerous cis-elements play a vital role in specifying the tissue expression patterns of plant genes. To understand what drives the expression of SIOFP20 in the pollen, a 2000-bp region upstream of the SIOFP20 start codon was submitted to a public database (http://www.dna.affrc.go.jp/PLACE) to analyze cis-acting elements to determine whether pollen development-associated elements were present in the promoter. There were ten pollen-specific activation-related elements and ten late pollen gene g10-related elements identified in the SIOFP20 promoter (Supplementary Table S2). This result further demonstrated that SIOFP20 may participate in pollen development.

Mild overexpression of SIOFP20 in tomato may promote seed germination

In this study, the plants with mild overexpression of SIOFP20 could generate fruits with seeds. However, compared to WT, the seed number of OE3 transgenic plants...
was reduced by ~49%, which may be partially due to the reduced male fertility of SIOFP20-OE transgenic plants (Fig. 12a). As the seed number was decreased in the transgenic plants, we sought to determine the germination energy of transgenic tomato seeds; thus, a seed germination experiment was performed. The results indicated that SIOFP20-OE transgenic seeds exhibited notably higher germination rates than those of WT (Fig. 12b, c), implying that mild overexpression of SIOFP20 in tomato may speed up transgenic tomato seed germination.

Discussion

The OVATE gene is initially found in tomato and shown to act as a plant-growth suppressor\textsuperscript{5}. Extensive studies have since focused on the functional analysis of this family in Arabidopsis and rice. As this research has progressed, OVATE family proteins have come to be considered vital regulators involved in organ shape and size determination\textsuperscript{5,9}, secondary cell wall formation\textsuperscript{10}, embryo sac development\textsuperscript{19}, fruit ripening\textsuperscript{16,17}, vasculature development\textsuperscript{12}, vegetative to reproductive phase transition\textsuperscript{11}, male transmission and pollen function\textsuperscript{6}, and phytochrome signaling\textsuperscript{7,13–15}. However, since the functional description of OVATE, little attention has been paid to the other OVATE family proteins in tomato. SIOFP20, a member of the OVATE family proteins, was isolated and functionally studied by using overexpression technology. Overexpression of SIOFP20 in wild-type tomato distinctly impacted the development of the vegetative and reproductive phases. In this paper, we were particularly concerned with the influence of SIOFP20 on the regulation of reproductive development. The strong overexpression of SIOFP20 in tomato resulted in exserted stigmas, an altered floral architecture and an absence of fruit, indicating that the overexpression of SIOFP20 affected the development of reproductive processes.

Overexpression of OVATE produces pleiotropic phenotypes in tomato plants, including exserted stigmas,
smaller floral organs, and round fruit\textsuperscript{5}. In our study, the floral architecture of the strong \textit{SIOFP20} overexpression lines resembled that of \textit{OVATE} overexpression lines. Similar phenotypes related to flowers have been found in \textit{Arabidopsis}\textsuperscript{7} and rice\textsuperscript{15}, indicating that OFPs exhibit overlapping functions in controlling plant growth and development. Statistical data suggested that the sepals, petals, and stamens of \textit{SIOFP20}-OE plants were much shorter and wider than those of WT plants. The exserted stigma phenotype of strong \textit{SIOFP20}-OE plants may be
ascribed to uneven repression of the growth of different floral organs, similar to what is observed upon the overexpression of \textit{ovate} in tomato.

BRs are a group of polyhydroxylated steroid hormones that play central roles in controlling plant growth and development and conveying various environmental inputs \cite{4,5,6}. The primary BR signaling pathway has been well established in \textit{Arabidopsis}. Briefly, BRs directly bind to BRI1 (BRASSINOSTEROID-INSENSITIVE1), which belongs to the plasma-membrane-localized and leucine-rich repeat (LRR) receptor kinases \cite{56,57,58}, thereby activating it and triggering a signal transduction cascade. Finally, the accumulation of unphosphorylated transcription factors BZR1/ BES1 in the nucleus modulates BR-responsive genes by binding target DNA \cite{59,60,61,62}. OsOFP1 \cite{14}, OsOFP8 \cite{13}, and OsOFP19 \cite{15} have been proposed to take part in the regulation of BR signaling. Considering the similar phenotypes observed in \textit{SIOFP20-OE} tomato plants and \textit{OsOFP19-OE} rice plants, we wondered whether the regulatory mechanisms of \textit{SIOFP20} and OsOFP19 affecting plant growth and development were conserved in tomato and rice. Ectopic expression of the rice homeobox gene \textit{OSHI} in tobacco generates thicker and shorter leaves \cite{54}, and loss-of-function of \textit{DWARF AND LOW-TILLERING (DLT)} results in a semidwarf phenotype with wider and shorter leaves \cite{65}. OsOFP19 directly interacts with OSH1 to increase the transcriptional activity of OSH1, resulting in a transition of the cell division pattern and antagonizing DLT in BR signaling, which positively regulates BR signaling \cite{15}. Thus, we attempted to examine the transcript levels of the homologous genes of \textit{OSHI} and \textit{DLT} in tomato, \textit{KNOXI} and \textit{GRAS41}. The results suggested that the mRNA accumulation of \textit{KNOXI} in \textit{SIOFP20-OE} transgenic lines was sharply increased. In contrast, the mRNA accumulation of \textit{GRAS41} was notably decreased. We also tried to examine the interactions between \textit{SIOFP20}, \textit{KNOXI}, and \textit{GRAS41} by using a yeast two-hybrid assay, and positive results were obtained. \textit{KNOXI} and \textit{GRAS41} exhibited a clear interaction, indicating that \textit{SIOFP20}, \textit{KNOXI}, and \textit{GRAS41} can form a complex. To determine the influence of the overexpression of \textit{SIOFP20} on BR metabolism, we assessed the expression of \textit{CPD}, \textit{D2}, and \textit{DWARF}, which are key genes for BR biosynthesis. The expression of \textit{CPD}, \textit{D2}, and \textit{DWARF} was significantly promoted in \textit{SIOFP20-OE} transgenic plants. In addition, we evaluated the transcript abundance of \textit{CYP734A7}, encoding a key BR catabolic enzyme. The transcript level of \textit{CYP734A7} was also increased in \textit{SIOFP20-OE} transgenic plants. These results are consistent with observations made in \textit{OsOFP19-OE} transgenic plants. In addition, we noted that the upregulation of BR biosynthesis genes may be due to feedback regulation by BR signaling, which was not discussed in relation to \textit{OsOFP19-OE} transgenic plants. \textit{OsOFP19-OE} plants show greatly reduced sensitivity to 24-epibraRassinolide treatment, indicating that \textit{OsOFP19} plays a negative role in the BR response \cite{15}. In rice, OSH1 promotes the mRNA accumulation of BR degradation-related genes, and inductive overexpression of \textit{OSH1} results in insensitivity to BR \cite{35,36}. Previous studies have suggested that BR-insensitive mutants exhibit increased transcript abundance of BR biosynthesis genes and a higher BR content \cite{35,36}. BRI1 is essential for the homeostasis of endogenous BR contents \cite{35}. Therefore, we measured the transcript level of \textit{BRI1}, which was notably increased in \textit{SIOFP20-OE} transgenic plants. Similar characteristics are found in the rice \textit{dlt} mutant, which also shows less sensitivity to BRs. The BR biosynthesis genes \textit{D2}, \textit{D11}, \textit{OsCPD}, and \textit{OsBr6ox} are all upregulated, as is the BR signaling gene \textit{BRI1} \cite{35}. In our study, the \textit{GRAS41} gene homolog of \textit{DLT} exhibited reduced levels in transgenic plants. Whether \textit{KNOXI} and \textit{GRAS41} participate in BR signaling has not yet been reported in tomato. Based on the above results, we speculate that \textit{SIOFP20} may negatively regulate the BR response in tomato, similar to \textit{OsOFP19} in rice. In addition, the altered floral architecture of \textit{SIOFP20-OE} plants may mainly be due to the reduced BR response.

The phenotypes of BR-deficient or BR-insensitive mutants are similar to those of GA-deficient or GA-insensitive plants. Many studies have focused on the question of whether BRs may regulate growth by impacting GA biosynthesis. BR treatment and overexpression of \textit{DWF4} increase the expression of three GA biosynthesis genes, \textit{GA20ox1}, \textit{GA20ox2} and \textit{GA20ox5}, in \textit{Arabidopsis} \cite{66}. In addition, BRs have been found to modulate GA biosynthesis in rice \cite{66}. Hence, we assumed that GA metabolism was altered in the \textit{SIOFP20-OE} transgenic lines. To investigate this hypothesis, the expression levels of GA biosynthesis genes, including \textit{CPS}, \textit{KS}, \textit{KAO}, \textit{GA20ox1}, and \textit{GA3ox1} and \textit{GA3ox2}, were measured. All of these genes showed increases in \textit{SIOFP20-OE} transgenic plants. Moreover, we detected the mRNA abundance of the GA inactivation genes \textit{GA2ox1} and \textit{GA2ox2}. The expression of \textit{GA2ox1} and \textit{GA2ox2} was increased. Interestingly, the mRNA accumulation of all of the examined BR and GA metabolism genes was found to be increased in our study. Previous work has shown that high levels of BRs induce GA inactivation by increasing the expression of \textit{GA2ox3} to counter the increase in GA biosynthesis due to increased \textit{GA3ox-2} expression, finally resulting in growth inhibition \cite{66}. As stated above, BR-insensitive mutants exhibit a higher content of BR, and \textit{SIOFP20} may negatively control BR signaling. Thus, we speculate that overexpression of \textit{SIOFP20} in tomato reduces BR signaling, resulting in the accumulation of BR, which then induces the mRNA accumulation of the GA inactivation genes \textit{GA2ox1} and \textit{GA2ox2} to counteract the increase in GA biosynthesis due to increased expression of \textit{CPD}, \textit{KS}, \textit{KAO}, \textit{GA20ox1}, \textit{GA3ox1}, and \textit{GA3ox2}, ultimately leading to growth inhibition.
There are hundreds of target genes downstream of the BR and GA pathways, including cell division and cell elongation; thus, the expression levels of cell division genes (CDKA1, SlCycA3;1, SlCycB1;1, SlCycB2, SlCycD2;1, E2FA, and SlCYCT1;3) and cell elongation genes (PRE1-5) were investigated. Four cell division genes, CDKA1, SlCycA3;1, SlCycB2, and SlCycD2;1, were significantly suppressed in the SIOFP20-OE transgenic plants. Overexpression of SlPRE2 in tomato promotes the elongation of plant stem internodes. In our study, the downregulation of SlPREs illustrated that cell elongation may be impaired in SIOFP20-OE transgenic plants. The SEM observations of the stamen surface also supported this result. Therefore, overexpression of SIOFP20 repressed cell division and cell elongation.

The key yield components of most crop species are fruit and seeds. Therefore, extensive studies have focused on fruit and seed development for decades. In our study, transgenic tomato plants with strong overexpression of SIOFP20 bore no fruit. BR-deficient and BR-perceptional mutants, including pdp, dwf4, and bri1, are male sterile or show significantly reduced male fertility due to shortening of the stamen and defects in pollen development, and these developmental defects correlate with the inhibition of several critical genes that participate in the development of anthers and pollen, indicating that BRs are critical for plant reproductive development. As mentioned above, overexpression of SIOFP20 reduced the BR response. Here, we also observed reduced length of the stamen, resulting in an exerted stigma phenomenon, which blocked the normal pollination process. On the other hand, a pollen germination experiment was carried out to examine the germination ability between WT plants and SIOFP20-OE transgenic tomato plants. The results showed that the pollen germination rate of the transgenic plants was significantly decreased, and this result was further supported by TTC staining, indicating that overexpression of SIOFP20 inhibits the normal development of pollen grains, but their form was not significantly altered when observed by SEM. In addition, a manual crossing assay was performed to verify that the maternal fertility of SIOFP20-OE transgenic lines was not affected. Furthermore, RT-qPCR analysis was conducted to check the transcript accumulation of tomato pollen-associated genes, including SICRK1, SIPMEI, LePRK3, SlPRALF, and LAT52. All of these genes showed a trend of downregulation in the transgenic lines, suggesting that SIOFP20 may control the mRNA accumulation of these pollen-specific genes to impact pollen development. In addition, previous studies revealed that many pollen-specific cis-acting elements, including the pollen-specific activation-related elements POLLEN1LELAT52 and the late pollen gene g10-related elements, were enriched in the promoter regions of two pollen-specific genes, SICRK1 and SIPMEI. Promoter-GUS chimeric expression experiments have been used to confirm that the promoters of SICRK1 and SIPMEI exhibit strong pollen-specific activity in the transgenic Arabidopsis and tomato plants. In our study, 10 pollen-specific activation-related elements POLLEN1LELAT52 and 10 late pollen gene g10-related elements were found in the SIOFP20 promoter, which further suggests that SIOFP20 may play an important role in pollen development. Similarly, AtOFP1 has been demonstrated to be essential for pollen function.

Moreover, tomato plants with mild overexpression of SIOFP20 can bear fruit. However, the seed number per fruit in the transgenic plants is sharply reduced compared with that in WT plants, which is in accord with the impairment of the pollen germination rate in transgenic plants. In addition, a seed germination experiment was performed to assess the quality of SIOFP20-OE transgenic seeds, and the results clearly showed that the germination rate of transgenic plants was higher than that of WT. AtOFP5 has been demonstrated to interact with BLH1 and KENAT3, which guarantees normal embryo sac development in Arabidopsis. Therefore, we assumed that SIOFP20 may participate in the development of the embryo sac, and more elaborate experiments should be performed to verify this possibility in the future.

In conclusion, the major objectives of this study were to investigate the influence of the overexpression of SIOFP20 on the reproductive development of tomato. Overexpression of SIOFP20 in tomato not only altered the morphology of the flowers and fruit but also reduced male fertility. In addition, mild overexpression of SIOFP20 may accelerate the germination rate of transgenic lines. Analyses of morphological, physiological, and molecular features have been performed to preliminarily elucidate the causes of SIOFP20-OE plants defects. A working model is proposed to explain the functions of SIOFP20 in plant growth and development (Fig. S3). Briefly, strong overexpression of SIOFP20 may directly inhibit the BR response via promoting the expression of KNOXI and suppressing the expression of GRAS41, leading to changes in a large number of genes, such as genes affecting cell division, cell elongation and pollen development. On the other hand, the reduced BR response in SIOFP20-OE plants leads to the accumulation of BR, which then induces the expression of GA inactivation genes to counteract the increase in GA biosynthesis. These results highlight that SIOFP20 functions as an important transcription factor to modulate reproductive development in tomato plants. Thus, it is meaningful to identify the biological function of SIOFP20 or other OVATE family proteins, which will not only extend knowledge of the biological functions of OVATE family proteins but also...
provide new insight for exploring the importance of OFPs in controlling plant vegetative growth and reproductive development. Considering that OFPs have been suggested to share overlapping functions with other OFP family members, a gain-of-function approach was used to study the functions of SlOFP20 in this study. In future studies, CRISPR/Cas9 genome editing may be a better way to generate SlOFP20 mutants for gene functional studies. To identify the protein partners and targeted genes of SlOFP20, it can be beneficial to elucidate the molecular mechanisms by which SlOFP20 modulates plant growth and development.

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

Supplementary Information
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