

The R2R3-MYB transcription factor CIMS188 targets *CIMS1L1* to orchestrate tapetum development and pollen formation in watermelon

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Abstract

Male-sterile lines are crucial genetic materials for investigating the developmental and abortion mechanisms of anthers, and they serve as key parental lines in hybrid seed production. However, research to date has primarily focused on model plants such as *Arabidopsis*, rice, and maize, whereas the regulatory mechanisms underlying this process remain largely unexplored in Cucurbitaceae. Here, we identified an R2R3-MYB transcription factor, CIMS188, as a key regulator of tapetum and pollen development. The *clms188* mutants exhibited complete male sterility due to aberrant tapetum development and tetrad formation. *In vitro* and *in vivo* experiments demonstrated that CIMS188 directly binds to the TAACCA motif in the *CIMS1L1* promoter and activates its transcription. Similarly, loss-of-function mutants of *CIMS1L1* led to defective tapetum and microspore development. Comparative transcriptomic analyses revealed that the expression of key genes in hormone signal transduction and phenylpropanoid biosynthesis pathways was altered in both the *clms188* and *clms1l1* mutants. Furthermore, the *clms188* mutant exhibited disruptions in sporopollenin precursor metabolism and tetrad wall degradation, whereas the *clms1l1* mutant showed alterations in genes for pollen coat precursor biosynthesis. Together, these findings establish the CIMS188–CIMS1L1 regulatory module that coordinates tapetum development and pollen formation, and provide a valuable genetic resource for hybrid seed production in watermelon.

Keywords: CIMS188, CIMS1L1, Tapetum and pollen development, Male sterile, Watermelon

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Introduction

Watermelon (*Citrullus lanatus*) is valued for its high nutritional content and health benefits, ranking as the third-largest fruit crop worldwide^[1,2]. Recent advances in genomic sequencing and functional characterization of watermelon have provided a solid foundation for molecular-assisted breeding, allowing more efficient integration of target traits^[3–6]. Owing to strong heterosis, commercial watermelon production relies heavily on F₁ hybrids. Male-sterile lines offer an efficient alternative to manual or chemical emasculation, reducing hybrid seed production costs while ensuring seed purity^[7], making them a key technology for harnessing heterosis. However, research on the molecular mechanisms underlying male sterility and the regulation of anther development in watermelon remains limited, which hinders the further advancement of the seed production industry. A total of nine male-sterile lines have been reported in watermelon, including the glabrous male-sterile (*gms*)^[8], male sterile 1 (*ms-1*)^[9], male sterile 2 (*ms-2*)^[10], male-sterile dwarf (*ms-dw*)^[11], male sterile 3 (*ms-3*)^[12], DAH3615-MS^[13], Se18^[14], *clapex1*^[15], and *clms1*^[5]. Among these, only the spontaneous mutant Se18 has been thoroughly studied, in which defective tapetal development during early anther formation leads to male sterility^[14].

The tapetum is crucial for pollen development^[16]. During early stages, it secretes nutrients for microspores. Later, it undergoes programmed cell death (PCD) to support pollen wall formation. Abnormal tapetum development or PCD can cause pollen abortion^[17]. Studies of anther development in *Arabidopsis* have identified five key transcription factors regulating tapetal development:

DYSFUNCTIONAL TAPETUM 1 (*AtDYT1*), DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION 1 (*AtTDF1*), ABORTED MICROSPORE (*AtAMS*), MALE STERILE 188 (*AtMS188*), and MALE STERILITY1 (*AtMS1*). These form a conserved *AtDYT1-AtTDF1-AtAMS-AtMS188-AtMS1* transcription cascade that precisely regulates tapetum and pollen development^[17–20]. Similar genetic pathways exist in rice (*OsUDT1-OsTDF1-OsTDR-OsMS188-OsPTC1*) and maize (*ZmMS32-ZmMS9-ZmHLH51-ZmMYB84-ZmMS7*)^[17,21–23].

AtMS188 (MALE STERILE 188, MYB103, MYB80) encodes an R2R3-MYB transcription factor and is expressed at the late stage of tapetum development, with its peak at the tetrad stage^[19,24]. Mutations in *AtMS188* result in abnormal tapetal PCD, defective callose degradation, and defective pollen exine formation^[25–29]. *AtMS188* binds to the promoter of *AtUNDEAD* to regulate tapetal PCD^[27]; additionally, *AtMS188* regulates *AtQRT3* expression for pectin degradation of the tetrad wall^[28], and together with *AtTEK*, co-regulates *AtA6* and its homologs (*AtA6s*) expression for callose degradation of the tetrad wall^[29]. Moreover, *AtMS188* directly binds to the promoters of genes related to sporopollenin synthesis, such as *AtPKSA*, *AtPKSB*, *AtMS2*, *AtCYP703A2*, *AtCYP704B1*, *AtACOS5*, and *AtTKPR1*, which promote pollen exine formation^[30,31]. Furthermore, *AtMS188* directly activates *AtMS1* to form a regulatory cascade where *AtMS1* controls the synthesis of pollen coat proteins (PCPs)^[32].

AtMS1 (MALE STERILITY1) encodes a PHD-finger transcription factor that is highly expressed in the tapetum at stage 9 (haploid microspores are released from tetrads) of anther development^[19]. Loss of function of *AtMS1* results in abnormal tapetal PCD and defective pollen exine and pollen coat formation^[25,32–34]. In *Arabidopsis*,

AtMS1 regulates the expression of both pollen coat protein genes (*AtGRP14*, *AtGRP17*, *AtGRP18*, *AtGRP19*, *AtEXL4*, and *AtEXL6*) and key genes for pollen coat lipid synthesis (*AtKCS7*, *AtKCS15*, and *AtKCS21*)^[32,35].

Unlike predominantly self-pollinated model plants (e.g., *Arabidopsis*, rice) or wind-pollinated crops (e.g., maize), watermelon is a typical insect-pollinated crop that relies heavily on animal vectors for reproduction^[36]. This strategy requires the formation of a specialized, adhesive pollen coat containing abundant lipids, phenols, and proteins to facilitate adherence to insect bodies. While the core *DYT1-TDF1-AMS-MS188-MS1* pathway is genetically conserved, it remains largely unknown how this regulatory cascade has evolutionarily adapted to coordinate the specific metabolic fluxes required for insect-mediated pollination in Cucurbitaceae. Previous research on the male-sterile line Se18 revealed that a conserved *CIDYT1-CITDF1-CIAMS-CIMS188-CIMS1L1* genetic pathway may be present in watermelon anther development^[14,37].

In this study, we employed CRISPR/Cas9 technology to generate *CIMS188* and *CIMS1L1* knockout lines, both of which exhibited complete male sterility. Using qRT-PCR and RNA *in situ* hybridization, we demonstrated that *CIMS188* and *CIMS1L1* are specifically expressed in anther tapetal cells, with their expression peaking at developmental stages 8b and 9, respectively. Sectioning results further revealed that the *clms188* and *clms1l1* mutants exhibit abnormal tapetal and microspore development, with abnormalities initiated at stages 8b and 9, respectively. Further *in vitro* and *in vivo* experiments demonstrated that *CIMS188* directly activates *CIMS1L1* expression. Transcriptome data revealed altered expression of multiple metabolic pathway genes and anther development regulatory genes in *clms188* and *clms1l1* mutants. This study investigates the *CIMS188*–*CIMS1L1* regulatory network for watermelon anther and pollen development, advancing knowledge of anther development mechanisms and providing gene resources for hybrid seed production.

Materials and methods

Plant materials and growth conditions

The WT watermelon inbred line YL was used for experimentation and genetic transformation in this study. Seeds were incubated at 28 °C in darkness for 3 d to induce germination, and the germinated seedlings were then transplanted into soil and grown until the third true leaves were fully expanded (16 h light, 28 °C/8 h dark, 20 °C, 50% relative humidity). After that, the plants were transferred to the Yangling Experimental Base of Northwest A&F University.

Phylogenetic analysis

Evolutionary analysis was conducted using the neighbor-joining method in MEGA 7.0, with 1,000 bootstrap replicates implemented^[38]. Multiple sequence alignments of *CIMS188*, *CIMS1L1*, and other homologous proteins were constructed using GENEDOC software. Sequence accession numbers are listed in [Supplementary Table S1](#).

Construction of gene editing vector and watermelon transformation

To generate the gene-edited mutants of *CIMS188* and *CIMS1L1*, we designed two sgRNAs targeting exons of each gene using CRISPR-P v2.0 (<http://crispr.hzau.edu.cn/CRISPR2>). We amplified the fragment containing both sgRNA sequences from the pCBC vector

by PCR, and then inserted it into the CRISPR/Cas9 vector pBSE402 through homologous recombination^[39]. The recombinant vector was transformed into *Agrobacterium tumefaciens* strain EHA105. The watermelon cotyledon explants were infected with the *Agrobacterium* strain, then subjected to co-cultivation, recovery culture, and selection on antibiotic-containing medium to obtain transgenic plants. After this, GFP fluorescence-positive plants were identified using a handheld fluorescence lamp (LUYOR-3415RG, USA). High-throughput tracking of mutations (Hi-TOM) and next-generation sequencing were employed to validate gene-edited plants^[40]. Independently derived homozygous T3 gene-edited mutant lines were used for subsequent studies. All the primers used in this experiment are listed in [Supplementary Table S2](#).

RNA *in situ* hybridization of male anther

Sense and antisense RNA probes for *CIMS188* and *CIMS1L1* were designed from their cDNA sequences and labeled with the DIG RNA Labeling Kit (Roche, Switzerland) following the manufacturer's instructions. WT male floral buds of varying diameters were collected and fixed in 4% paraformaldehyde at 4 °C for 24 h. After dehydration, the samples were infiltrated with paraffin using xylene at 42 °C, allowed to cool naturally, and then embedded and sectioned. The samples were pre-hybridized in pre-hybridization buffer at 42 °C for 3 h, followed by overnight hybridization at 42 °C with the addition of DIG-labeled RNA probes. Finally, the color was developed by incubation with BCIP/NBT solution, and the signals were observed^[40]. All the primers used in this experiment are listed in [Supplementary Table S2](#).

Semi-thin resin sectioning

WT and mutant male floral buds of varying diameters were collected and fixed in FAA solution at 4 °C for 24 h. Samples were then pre-infiltrated in a 1:1 mixture of 100% ethanol and 7,100 base liquid for 2–3 d. Subsequently, 1 g of hardener I diluted in 100 mL of 7,100 base liquid was added to the samples, followed by incubation at 4 °C for 2–3 d. For polymerization, 1 mL of hardener II was diluted in 15 mL of osmotic solution, and the resulting polymerization solution was added to each sample. After an additional polymerization period of 3 d, samples were longitudinally sectioned to 5 µm thickness using a semi-thin microtome (RM2265; Leica). Sections were stained with 1% TBO for 1 min, rinsed with running water, and heated on a hot plate for 2 h. Finally, sections were mounted in neutral resin and imaged under bright-field microscopy^[40].

Alexander staining of pollen

Male flowers of WT, *clms188*, and *clms1l1* mutants were collected on the same day. Pollen was gently tapped onto glass slides, stained with 100 µL Alexander staining solution, and then covered with coverslips. After staining for 5 min, pollen viability was observed under a microscope (Axio Imager M2; Zeiss) and documented photographically.

Dual-luciferase assay

The *CIMS1L1* promoter (–1,837 to –1 bp) was cloned into the pGreenII 0800-Luc vector. The *CIMS188* CDS was cloned into the pGreenII 62-SK vector^[41]. The recombinant vectors were separately transformed into *Agrobacterium* strain GV3101 (pSoup). *Agrobacterium* cells containing the constructed effector and reporter (effector : reporter = 9:1) were co-infiltrated into *N. benthamiana* leaves. The activities of LUC and REN were measured using a Dual-Luciferase

reporter kit (Yeasen Biotech, China) with five replicates. All the primers used in this experiment are listed in [Supplementary Table S2](#).

Yeast one-hybrid assay

The *CIMS188* CDS was cloned into the pGADT7 vector. The *CIMS1L1* promoter segment was divided into *CIMS1L1*-P1 (−1,837 to −1,092 bp) and *CIMS1L1*-P2 (−1,092 to −1 bp) fragments, which were cloned into the pAbAi vector. Transformed yeast strains were cultured on SD/-Leu medium with or without 80 ng/mL aureobasidin A (AbA) at 30 °C for 3 d, followed by observation and photography. All the primers used in this experiment are listed in [Supplementary Table S2](#).

Electrophoretic mobility shift assay

The *CIMS188* CDS was fused to an MBP-tag and cloned into the pMAL-C2X vector. This construct was introduced into *Escherichia coli* Rosetta, and expression was induced with 0.5 mM IPTG. The CIMS188-MBP fusion protein was purified using the LightShift™ Chemiluminescent EMSA Kit (Beyotime, China). Putative target gene promoter fragments were synthesized as biotin-labelled oligonucleotides. All the primers used in this experiment are listed in [Supplementary Table S2](#).

Subcellular localization

The *CIMS188* CDS and *CIMS1L1* CDS were fused to an eGFP tag and cloned into the pGreen vector to construct a *p35S::CIMS188-GFP* fusion expression vector. NLS-RFP was marked with AtH2B-mCherry. The recombinant vectors were separately transformed into *Agrobacterium* strain GV3101 (pSoup). *Agrobacterium* cells containing the constructed vectors were co-infiltrated into *N. benthamiana* leaves. After 72 h, GFP fluorescence in the injected leaves was observed under an Olympus BX63 microscope (Tokyo, Japan). All the primers used in this experiment are listed in [Supplementary Table S2](#).

Quantitative real-time PCR (qRT-PCR) assays

Around 10:00 AM, different tissues and florets of different diameters were collected from WT, *clms188*, and *clms1l1* plants and flash-frozen in liquid nitrogen. Total RNA was extracted with the RNA Simple Total RNA Kit (Tiangen, China), and cDNA was synthesized using the Fast King RT Kit (Tiangen, China). SYBR Green PCR Master Mix (Takara) was used for qRT-PCR. Relative expression levels were normalized against the watermelon β -ACTIN (Gene ID: *Cl97C02G026960*)^[42]. All primers used in this experiment are listed in [Supplementary Table S2](#).

Statistical analysis of RNA-seq data

The florets of WT, *clms188*, and *clms1l1* plants from stage 6 to stage 10 (bud diameter 2.0–4.5 mm; [Supplementary Tables S3](#)) were collected and flash-frozen in liquid nitrogen^[14]. For each sample, three biological replicates were collected from specified tissues from independent WT and mutant plants. RNA extraction, mRNA enrichment, RNA-seq library construction, and sequencing were carried out by Biomarker (Beijing, China). The bioinformatics analysis of RNA-seq data was performed using BMKCloud (www.biocloud.net). DEGs were screened according to the screening criteria of fold-change ≥ 2 and FDR < 0.01 . The enriched GO classification and KEGG pathways among the overlapping DEGs were identified using BMKCloud. General information regarding the RNA-seq data is listed in [Supplementary Tables S4–S10](#). All primers used in this experiment are listed in [Supplementary Table S2](#).

Results

Characterization of CIMS188 in watermelon

According to watermelon genomic annotation, the genomic sequence of *CIMS188* contains three exons and two introns; its 966-bp open reading frame (ORF) encodes a 321-amino-acid protein (≈ 35.5 kDa) ([Supplementary Fig. S1a, S1b](#)). Sequence alignment confirms CIMS188 contains a typical R2R3-MYB domain, classifying it as an R2R3-MYB transcription factor ([Supplementary Fig. S1c](#)). Phylogenetic analysis shows CIMS188 groups with orthologs from other plants, while its paralogs from *Arabidopsis* and watermelon form two separate clades ([Supplementary Fig. S1d](#)). CIMS188-GFP co-localized with a nuclear marker, confirming its nuclear localization as an R2R3-MYB transcription factor ([Fig. 1a](#)).

Based on the developmental staging criteria for watermelon anther development established in previous research^[14] ([Supplementary Table S3](#)), we analyzed the expression level of *CIMS188* in various tissues of the wild-type (WT) plant by quantitative real-time PCR (qRT-PCR) and RNA *in situ* hybridization. The qRT-PCR results showed that *CIMS188* was generally weakly expressed in vegetative organs (roots, stems, leaves, petioles, tendrils) and female flowers; however, its expression was significantly upregulated during male flower development, especially during stages 6–9, with a peak at stage 8b ([Fig. 1b](#)). RNA *in situ* hybridization showed that *CIMS188* transcript levels were low during stages 6–7 ([Fig. 1c](#)), followed by a steady increase in the tapetal cells and microsporocytes at stage 8a. At stage 8b (the tetrad stage), *CIMS188* expression reached its highest level specifically in the tapetum. During microgametogenesis, *CIMS188* transcript abundance decreased sharply in both the tapetum and microspores. These results suggest that *CIMS188* is specifically expressed during anther development, with the highest expression level at stage 8b, indicating that the transcription factor CIMS188 may play an important role in this process.

Knockout of CIMS188 results in complete male sterility

To further study the function of *CIMS188*, a dual-target CRISPR/Cas9 vector was constructed using sgRNAs designed to target the first and second exons ([Fig. 2a, b](#)). Three *CIMS188* mutant lines were obtained: *clms188-1* (5-bp deletion) and *clms188-2* (1-bp deletion), both of which resulted in frameshifts and premature termination codons, whereas *clms188-3* had a 131-bp deletion, leading to a partial loss of the R2R3-MYB protein domain ([Fig. 2b; Supplementary Fig. S2](#)). Compared with WT, all *clms188* mutants exhibited complete male sterility. For detailed phenotypic analysis, we selected *clms188-1* as a representative mutant ([Fig. 2c–j](#)). In WT plants, female flowers and male flowers at one-to-two-node lower positions opened synchronously. However, in the *clms188* mutants, male flowers at comparable nodes typically remained unopened, although female flowers at adjacent positions flowered normally ([Fig. 2c, d](#)). Notably, under low-temperature and low-light conditions, the *clms188* mutant occasionally had a single male flower with open petals; however, these flowers displayed significantly shorter petals, reduced anther diameter, and a complete absence of pollen grains ([Fig. 2e, f; Supplementary Table S11](#)). Stereomicroscopy showed that WT anthers dehisced normally, releasing abundant, adherent yellow pollen. In contrast, *clms188* anthers were indehiscent and lacked visible pollen ([Fig. 2g, h; Supplementary Table S11](#)). Alexander staining confirmed abundant viable pollen in WT, whereas no pollen was detected in *clms188* ([Fig. 2i, j; Supplementary Table S11](#)). To investigate whether the *clms188* mutation affects the growth and

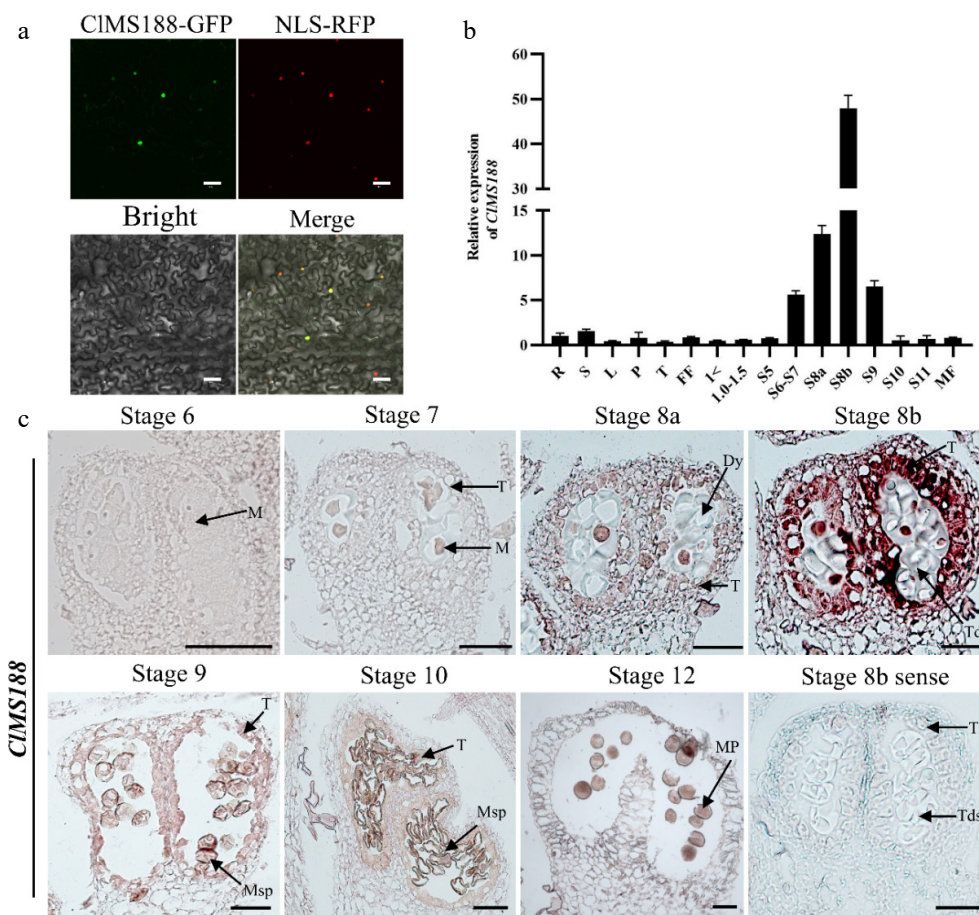


Fig. 1 Subcellular localization and expression analysis of the *CIMS188* gene. (a) Subcellular localization of *CIMS188*. The constructs *p35S::CIMS188-GFP* and *p35S::NLS-RFP* were transformed into *N. benthamiana* leaves and then observed by confocal microscopy. Scale bars, 50 μ m. (b) Transcript levels of *CIMS188* in the roots (R), stems (S), leaves (L), petioles (P), tendrils (T), female flowers (FF), male floral buds of different diameters, and open male flowers (MF) of WT plants. '> 1' indicates male floral buds with a diameter > 1 mm; 'S5' indicates male floral buds at stage 5 of anther development. Relative expression was normalized to the expression level in WT roots. Data are means $\pm$ SDs ($n = 3$). (c) RNA *in situ* hybridization of *CIMS188* transcript in WT anthers during stages 6–12 is shown. Stage 8b anthers hybridized with the sense probe served as the negative control. Dy, dyad cell; M, microsporocyte; Mp, mature pollen; Msp, microspore; T, tapetum; Tds, tetrads. Scale bars, 20 μ m.

development of female flowers, fruits, and seeds, we crossed the *clms188* mutant (as the female parent) with WT plants (as the male parent). The results showed that the *clms188* mutant exhibited no significant differences from WT in female flower opening, number of female flower nodes, fruit size, fruit weight, and seed number (Supplementary Fig. S3; Supplementary Table S11). In summary, our findings indicate that *CIMS188* is indispensable for male fertility, as its knockout results in complete male sterility without affecting other traits.

To further characterize the defective *clms188* mutant, semi-thin sections of anthers from WT and mutant plants were examined across developmental stages (Fig. 2k, l). No morphological differences were observed from microsporocyte formation (stage 6) through the completion of meiosis I and dyad formation (stage 8a). Starting from stage 8b, anther development in *clms188* began to show abnormalities. Specifically, at stage 8b, dyads in WT completed meiosis II and formed tetrads of four haploid microspores, and tapetal cells began to degrade. In contrast, *clms188* tetrads were abnormally shaped, with the microspores shrinking and showing irregular morphology, and tapetal cells were enlarged and deeply stained. At stage 9, WT microspores were released into the anther locules, the tapetum degenerated, and the callose was degraded; in *clms188*, microspores further shrank and became deformed, while

the tapetal cells expanded and underwent vacuolation. At stage 10, large vacuoles formed in WT microspores, squeezing the nucleus to the periphery; the microspore wall formed, and the tapetum further degraded; in *clms188*, microspores continued to shrink, and the tapetum became faintly stained and further enlarged. From stage 11 to stage 12, WT microspores underwent two mitotic divisions to form mature pollen filled with starch and lipids, and the tapetum completely degraded; in *clms188*, microspores were largely degraded, and the tapetum completely degenerated. Sectioning results showed that the *clms188* phenotype is caused by abnormal development of both the tapetum and microspores starting from stage 8b.

CIMS188 directly activates *CIMS1L1* expression

Studies in *Arabidopsis* have demonstrated that AtMS188 directly regulates the expression of AtMS1 by binding to its promoter^[27,32]. To determine whether CIMS188 directly regulates *CIMS1L1* expression during watermelon male flower development, a dual-luciferase (LUC) assay was conducted in *N. benthamiana* leaves with CIMS188 as the effector and a *CIMS1L1* promoter-driven construct as the reporter (Fig. 3a). Compared to the empty vector control, the LUC signals of *pCIMS1L1::LUC* were significantly upregulated by

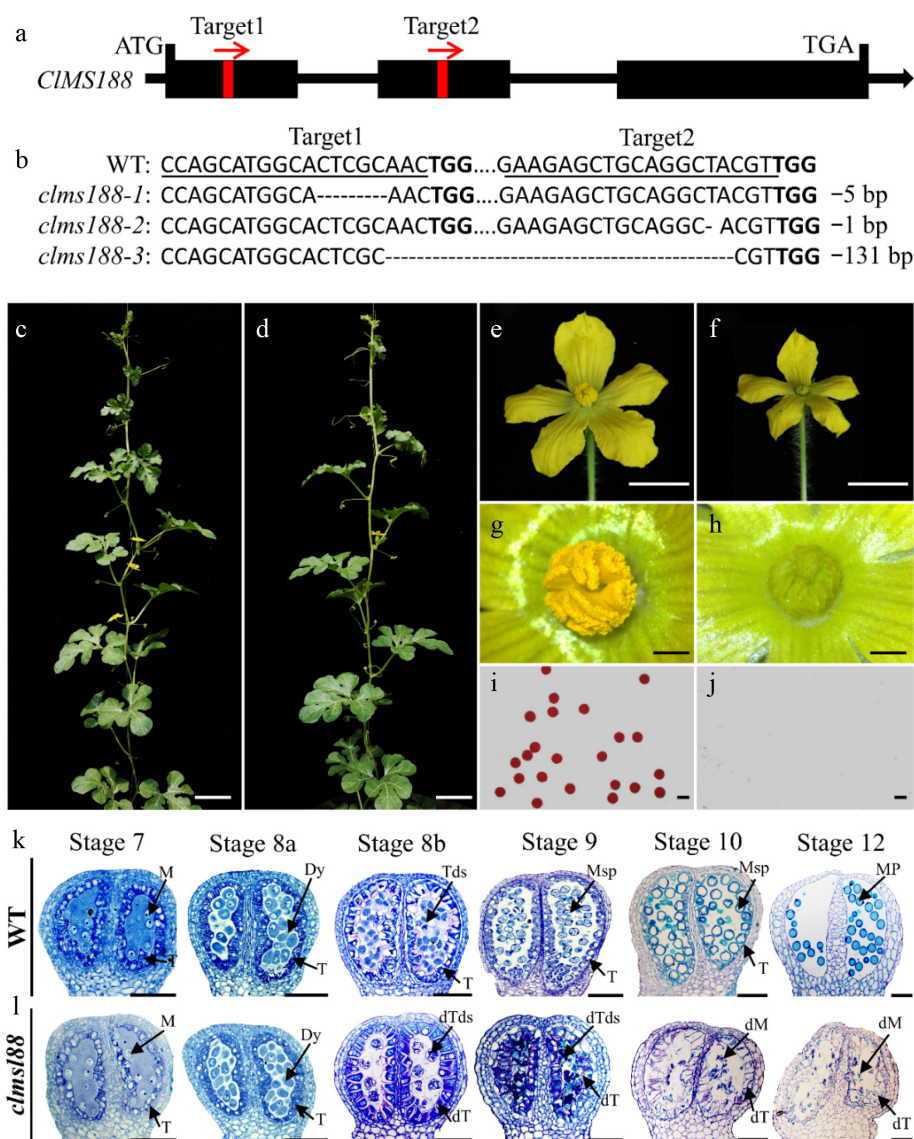


Fig. 2 Knockout of *CIMS188* to obtain completely male-sterile mutants. (a) Gene structure and target site positions of *CIMS188*. Black boxes represent exons; red boxes represent target site locations; red arrows represent target site orientation. (b) Mutation profiles of *CIMS188* in three mutant lines identified by sequencing. PAM motifs are in bold; target sites are underlined; '-' indicates deletions. Phenotypes of the (c) WT and (d) *clms188*. Scale bars, 10 cm. Male flower morphology of (e) WT and (f) *clms188*. Scale bars, 200 μ m. Alexander staining of pollen from (g) WT and (h) *clms188*. Scale bars, 60 μ m. Semi-thin sections of (i) WT and (j) *clms188* mutant anthers during stages 6–14 are shown. dM, defective microsporocyte; dT, defective tapetum; dTds, defective tetrads. Dy, dyad cell; M, microsporocyte; Mp, mature pollen; Msp, microspore; T, tapetum; Tds, tetrads. Scale bars, 20 μ m.

p35S::CIMS188 (Fig. 3b). These data indicate that *CIMS188* directly activates *CIMS1L1* expression. Previous studies demonstrate that *AtMS188* binds to cis-elements sharing a conserved AACC core sequence^[27,30–32]. Analysis of the *CIMS1L1* promoter (–1,837 bp to –1 bp) revealed eight AACC elements (Fig. 3c). To map the *CIMS188* binding region, we divided this promoter segment into two fragments (*CIMS1L1*-P1 and *CIMS1L1*-P2) and performed yeast one-hybrid (Y1H) assays (Fig. 3c, d). The Y1H assays preliminarily demonstrated that *CIMS188* specifically binds to the *CIMS1L1*-P1 region, which contains four AACC core sequences (Fig. 3d). To further identify specific binding sites within *CIMS1L1*-P1, an electrophoretic mobility shift assay (EMSA) was utilized. Four sets of probes containing AACC elements within *CIMS1L1*-P1 were synthesized, named Probe-1^{*CIMS1L1*} (AAACCC), Probe-2^{*CIMS1L1*} (TAAACC), Probe-3^{*CIMS1L1*} (TAACCA), and Probe-4^{*CIMS1L1*} (AAACCT) (Fig. 3e). EMSA

results showed that *CIMS188* bound specifically to Probe-3^{*CIMS1L1*}, but not to the other probes (Fig. 3e, f). These results demonstrate that *CIMS188* directly binds to the TAACCA motif in the *CIMS1L1* promoter and activates *CIMS1L1* transcription.

Loss of function of *CIMS1L1* leads to complete male sterility

To further elucidate the function of *CIMS1L1*, comprehensive bioinformatics characterization and expression profiling were conducted. The results show that the *CIMS1L1* gene consists of four exons and three introns, encoding a 649-amino-acid PHD-finger transcription factor (≈ 73.7 kDa) containing an N-terminal nuclear localization signal and a C-terminal PHD domain (Supplementary Fig. S4a–S4c). Phylogenetically, *CIMS1L1* belongs to the dicot clade of the MS1 family (Supplementary Fig. S4d). *CIMS1L1* localizes to the

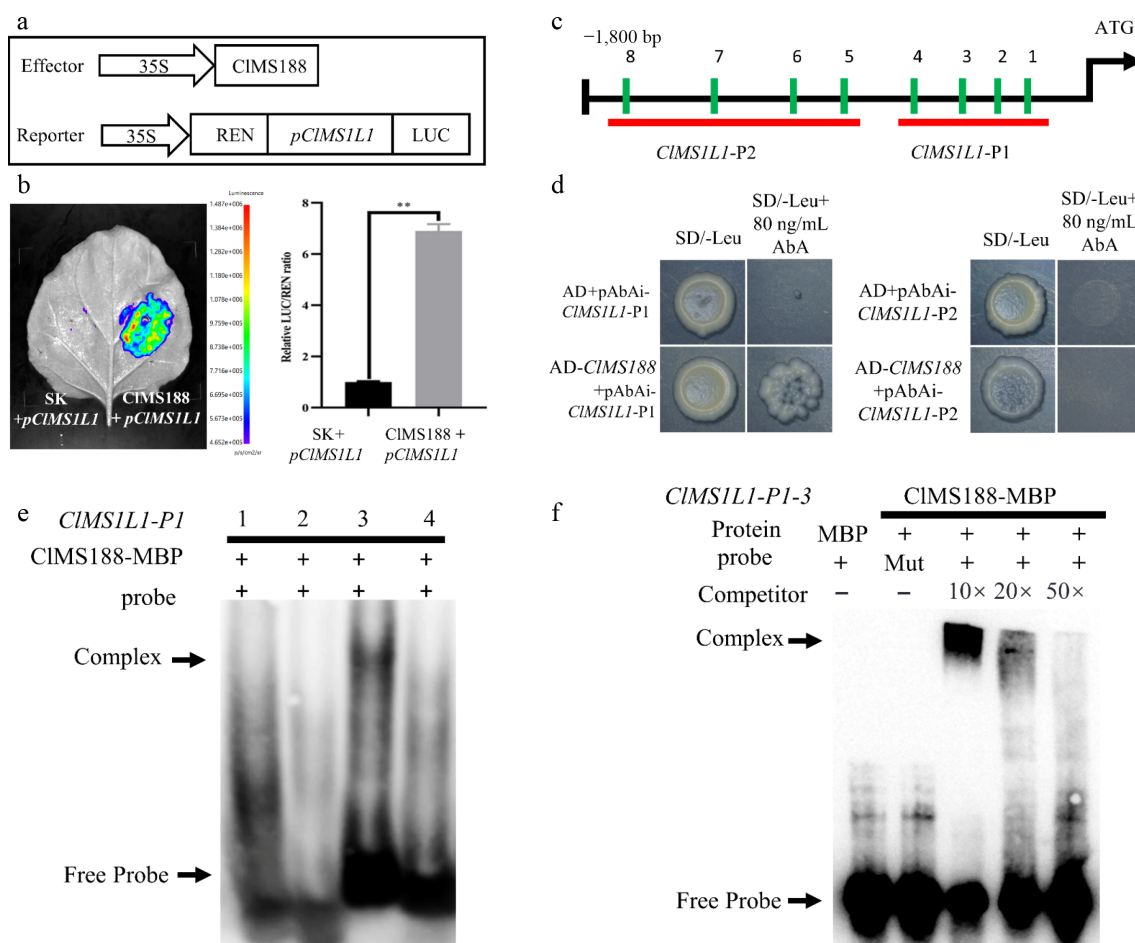


Fig. 3 CIMS188 binds to the promoter of *CIMS1L1* and activates its expression. (a) Schematic diagram of effector and reporter constructs for dual-luciferase assay. (b) Dual-luciferase assay to evaluate CIMS188-mediated transactivation of *CIMS1L1*. The data represent the mean $\pm$ SD of five independent experiments. ** Indicates significant differences at $p < 0.01$. (c) AACC elements in the upstream promoter region of *CIMS1L1*. (d) Yeast one-hybrid assay of CIMS188 binding to the *CIMS1L1* promoter. Yeast cotransformed with the empty vector pGADT7 (AD) and the *CIMS1L1* promoter was used as a negative control. Yeast cotransformed with pAbAi-*CIMS1L1*-P1 and pAbAi-*CIMS1L1*-P2, along with AD-*CIMS188* transformants, grew well on selective medium (SD/-Leu) supplemented with 80 ng/mL AbA. (e), (f) Identification of CIMS188 binding specificity to sites on the *CIMS1L1* promoter by electrophoretic mobility shift assays. (e) Binding verification of CIMS188 to four sets of probes, Probe-1^{CIMS1L1} (AAACCC), Probe-2^{CIMS1L1} (TAAACC), Probe-3^{CIMS1L1} (TAACCA), and Probe-4^{CIMS1L1} (AAACCT) on *CIMS1L1*-P1. (f) CIMS188 binding specificity to sites on the *CIMS1L1*-P1 promoter of Probe-3^{CIMS1L1} (TAACCA). '−' indicates absence; '+' indicates presence. The arrow indicates the position of the protein-DNA complex after incubation of a biotin-labelled DNA probe with MBP-MS188.

nucleus, as confirmed by fluorescence co-localization with a nuclear marker (Supplementary Fig. S4e). Both qRT-PCR and RNA *in situ* hybridization showed that *CIMS1L1* was weakly expressed at early stages of anther development, increased gradually in the tapetum and microsporocytes, peaked at stage 9, and then declined sharply, suggesting that *CIMS1L1* plays a crucial role during these stages (Fig. 4a, b). Furthermore, we examined the expression level of *CIMS1L1* in the *clms188* mutant background. In comparison with the WT, *CIMS1L1* expression was significantly lower in the *clms188* mutant at multiple anther developmental stages, further indicating that *CIMS1L1* acts downstream of *CIMS188* (Supplementary Fig. S4f). Next, we generated three independent *CIMS1L1* knockout lines using a dual-target CRISPR/Cas9 strategy targeting its third exon (Fig. 4c). The lines carried distinct mutant alleles: *clms111-1* (218-bp deletion) and *clms111-3* (4-bp deletion) result in frameshift and premature truncation, while *clms111-2* (219-bp deletion) had an in-frame deletion affecting part of the activation domain (Fig. 4d; Supplementary Fig. S5). All mutants exhibited male sterility. We selected *clms111-1* as the representative mutant for phenotypic

analysis (Fig. 4e). In the *clms111* mutant, male flowers (at nodes comparable to the wild type) remained closed (Fig. 4e). Occasionally, the *clms111* mutant produced a single male flower with shorter petals, smaller anthers, and no pollen (Fig. 4f; Supplementary Table S11). Stereomicroscopy and Alexander staining confirmed indehiscent anthers with no visible pollen (Fig. 4g, h). Genetic crosses also indicated that *clms111* mutants are male-sterile without pleiotropic effects on other traits (Supplementary Fig. S6; Supplementary Table S11).

To examine the phenotypic impact of the *clms111* mutation, we examined the anther and pollen morphology of WT and *clms111* at various anther stages (Fig. 4i). Sectioning revealed that *clms111* showed no visible differences from WT from microsporocyte formation (stage 6) through meiosis II and tetrad formation (stage 8b). At stage 9, the microspores of *clms111* were stained unevenly, shrank, and became deformed, while the cytoplasm of tapetal cells expanded, and the tapetum exhibited intense staining. At stage 10, the microspores further shrank and began to degrade, and the tapetum became abnormally enlarged, occupying most of the anther

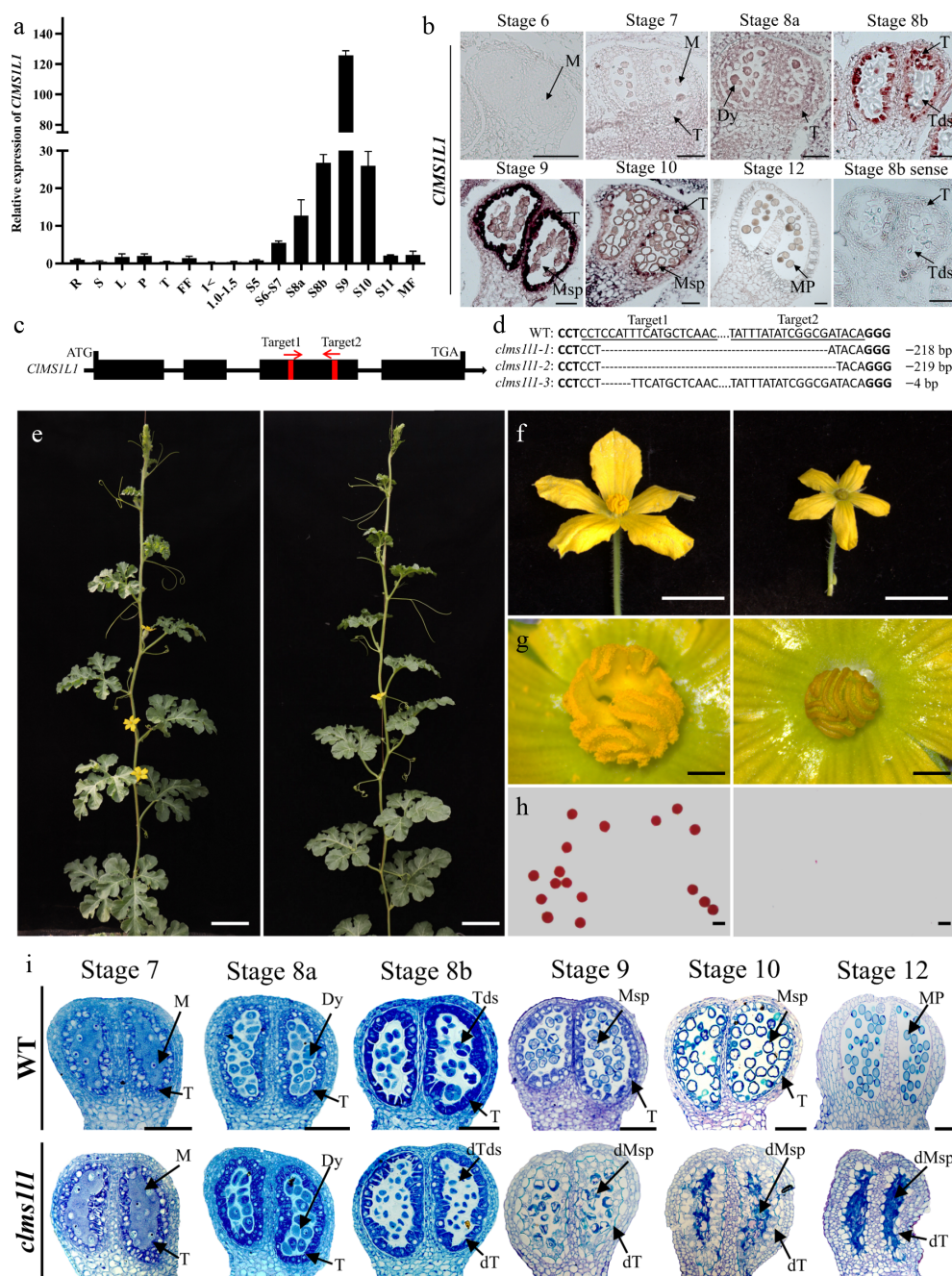


Fig. 4 Expression and function of the *CIMS1L1*. (a) Transcript levels of *CIMS1L1* in the roots (R), stems (S), leaves (L), petioles (P), tendrils (T), female flowers (FF), male floral buds of different diameters, and open male flowers (MF) of WT plants. (b) RNA *in situ* hybridization of *CIMS1L1* transcript in WT anthers during stages 6–12. (c) Gene structure and target site positions of *CIMS1L1*. (d) Mutation profiles of *CIMS1L1* in three mutant lines identified by sequencing. (e) Phenotypes of the WT and *CIMS1L1*. Scale bars, 10 cm. (f) Male flower morphology of WT and *clms1l1*. Scale bars, 1 cm. (g) Anther morphology of the WT and *clms1l1*. Scale bars, 200 μ m. (h) Alexander staining of pollen from WT and *clms1l1*. Scale bars, 60 μ m. (i) Semi-thin sections of WT and *clms1l1* anthers during stages 6–12. Scale bars, 20 μ m.

locules. From stage 11 to stage 12, the microspore fragments were largely degraded, and the tapetal cells remained expanded. In summary, sectioning results showed that the *clms1l1* mutation results in abnormal tapetal and microspore development initiated at stage 9.

Transcriptome analysis of *clms188* and *clms1l1* mutants

To delineate the regulatory networks of *CIMS188* and *CIMS1L1*, we conducted RNA-seq on anthers from WT, *clms188*, and *clms1l1*

plants during stages 6–10 (*clms1l1-1* and *clms188-1* were used as the representative mutants for transcriptome analysis). RNA-seq generated 206.7 million high-quality reads, revealing reproducible intra-genotype and distinct inter-genotype patterns (supported by PCC/PCA) and identifying DEGs ($|FC| \geq 2$, $FDR < 0.01$), 10 of which were validated by qRT-PCR (Supplementary Fig. S7; Supplementary Table S4). In total, 1,875 DEGs were identified in WT vs. *clms188* (917 upregulated and 958 downregulated), and 2,266 DEGs were identified in WT vs. *clms1l1* (1,282 upregulated and 1,004 downregulated) (Supplementary Fig. S8a, S8b; Supplementary Tables S5, S6).

Furthermore, 710 genes overlapped between the DEG lists from the WT vs. *clms188* and WT vs. *clms111* comparisons, of which 439 were co-downregulated, and 271 were co-upregulated (Supplementary Fig. S8c; Supplementary Tables S7, S8). The top 20 most enriched KEGG terms in the analysis of DEGs showed that, for WT vs. *clms188*, the DEGs were mainly involved in pathways such as plant hormone signal transduction (ko04075), phenylpropanoid biosynthesis (ko00940), pentose and glucuronate interconversions (ko00040), galactose metabolism (ko00052), and glycerolipid metabolism (ko00561) (Supplementary Table S9). For WT vs. *clms111*, the DEGs were mainly involved in plant hormone signal transduction (ko04075), plant-pathogen interaction (ko04626), phenylpropanoid biosynthesis (ko00940), starch and sucrose metabolism (ko00500), and pentose and glucuronate interconversions (ko00040) (Supplementary Table S9). Notably, both comparisons showed significant enrichment for plant hormone signal transduction (ko04075) and phenylpropanoid biosynthesis (ko00940) (Supplementary Fig. S8d, S8e).

We selected genes involved in auxin (IAA), gibberellin (GA), jasmonic acid (JA), and brassinosteroid (BR) signaling pathways of plant hormone signal transduction (ko04075) in both groups for further analysis (Fig. 5; Supplementary Fig. S9a; gene IDs see Supplementary Table S10). The selected genes include: auxin signaling-related genes, including *CILAX*, *CIAA* family genes, and *CISAUR* family genes; GA signaling-related genes, including *CIGID1*, *CISCL18*, and *CIPIF1*; JA signaling-related genes, including *CIJAR1* and *CI MYC2* family genes; BR signaling-related genes, including *CIBAK1* family genes, and *CIBR1* family genes, *CIBKI1*, *CITCH4*, and *CICYCD3* (Supplementary Fig. S10a). These genes likely play central roles as

hormone-related regulators in the *CIDYT1-CITDF1-CIAMS-CIMS188-CIMS111* genetic pathway. Furthermore, about half of the differentially expressed genes are specific to either the *clms188* or *clms111* mutant, suggesting that these genes are likely regulated by CIMS188 and CIMS111, respectively. These results align with established roles of these hormones in male flower development^[43–51].

Phenylpropanoid biosynthesis pathway (ko00940) produces compounds like lignins, coumarins, and flavonoids, which are essential for pollen and anther development (Supplementary Fig. S9b)^[52,53]. Pathway enrichment analysis identified 39 DEGs for WT vs. *clms188* and 66 DEGs for WT vs. *clms111* (Fig. 6a, b; Supplementary Table S10). Of these, 20 DEGs were common to both comparisons, including core phenylpropanoid biosynthesis pathway genes, such as *CIPAL* family genes, *CIC4H*, *CI4CL* family genes, and *CIHCT*; lignin biosynthesis pathway genes, such as *CICCR* family genes, and peroxidase family genes; and coumarin biosynthesis pathway genes, such as *CIGH1* family genes (Supplementary Fig. S10b; Supplementary Table S10). Notably, in comparisons involving the lignin and coumarin biosynthesis pathways, the WT vs. *clms188* group exhibited approximately twice as many DEGs as the WT vs. *clms111* group (Fig. 6a, b; Supplementary Table S10). This implies that CIMS111 might adopt a more intricate and specialized regulatory mechanism in these two pathways. Chalcones, serving as precursors in the flavonoid biosynthesis pathway (ko00941), can be further metabolized to flavonols through a series of enzymatic reactions. The key genes of this pathway, such as *CICH5* and *CIFLS*, were enriched (Fig. 6b; Supplementary Table S10). In conclusion, we hypothesize that both CIMS188 and CIMS111 play important regulatory roles in the phenylpropanoid biosynthesis and related metabolism pathways.

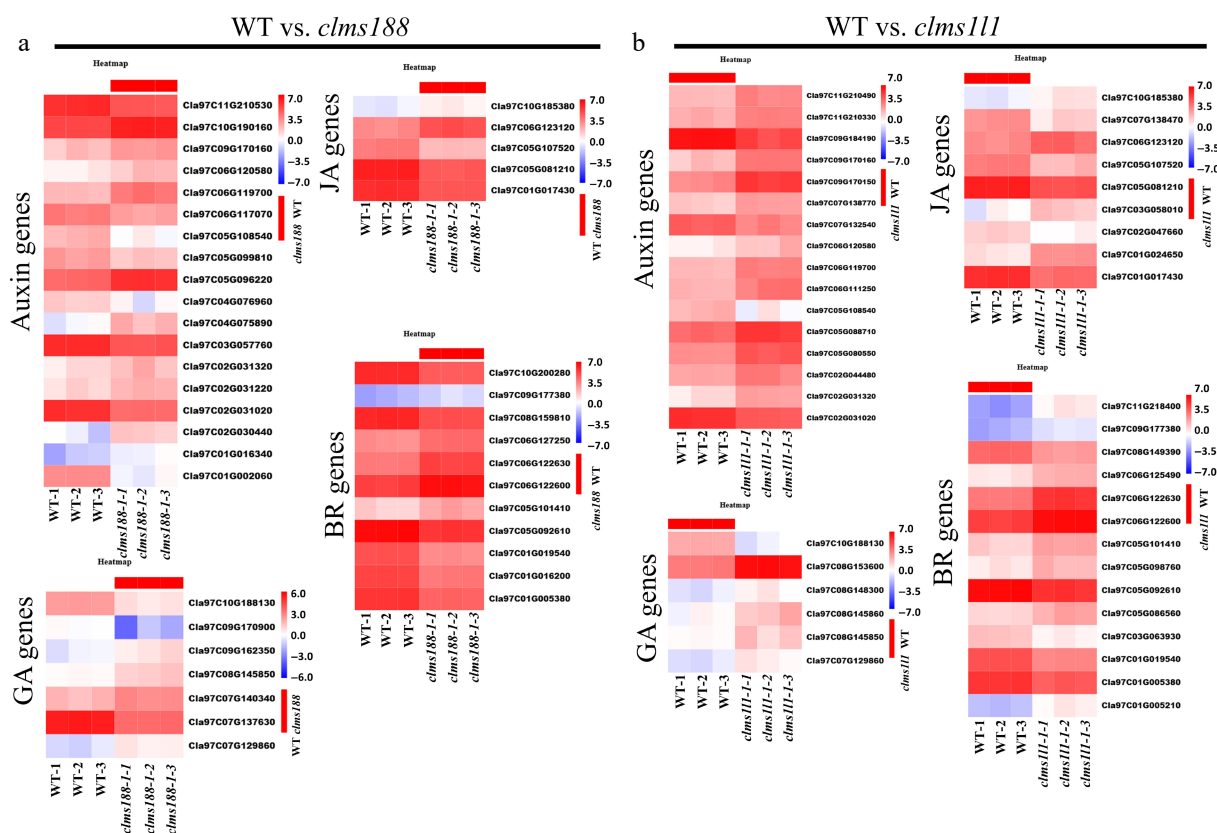


Fig. 5 KEGG enrichment of plant hormone signal transduction pathway in WT vs. *clms188* and in WT vs. *clms111*. Heatmaps showing the expression of DEGs associated with auxin, gibberellin, jasmonic acid, and brassinosteroid pathways in (a) WT vs. *clms188* and (b) WT vs. *clms111*. The color bar on the right represents expression levels, with red and blue indicating high and low expression, respectively.

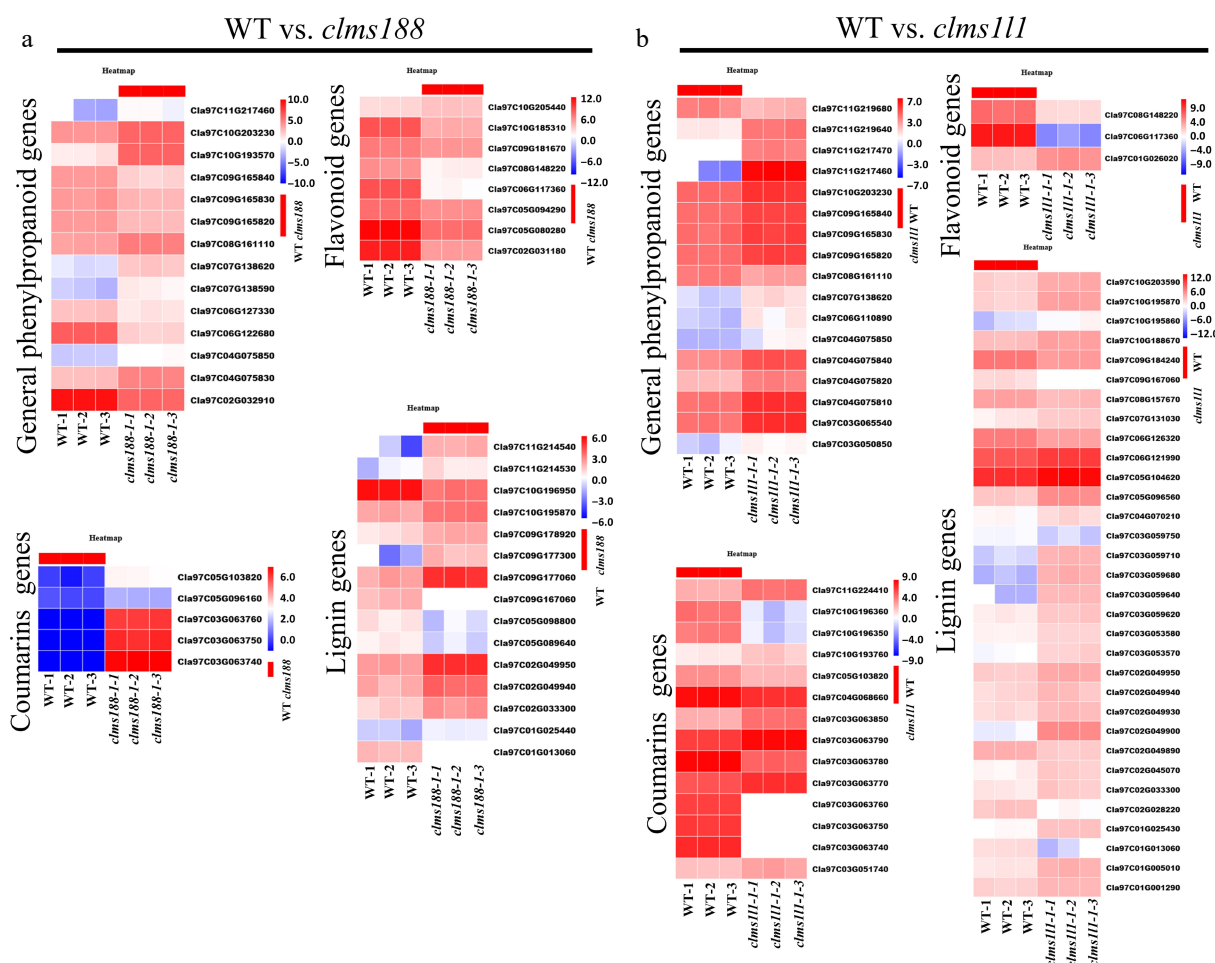


Fig. 6 KEGG enrichment of phenylpropanoid-related pathways in WT vs. *clms188* and in WT vs. *clms111*. (a), (b) Heatmaps showing the expression of DEGs related to general phenylpropanoid biosynthesis, lignin, coumarin, and flavonoid metabolism in (a) WT vs. *clms188* and (b) WT vs. *clms111*. The color bar on the right represents expression levels, with red and blue indicating high and low expression, respectively.

Identification of potential male sterility-related genes via DEGs analysis in *clms188* and *clms111* mutants

Genetic evidence indicates a shared pathway for the synthesis of phenylpropanoids, sporopollenin, and tryphine^[54,55]. It has been reported that 13 genes are involved in sporopollenin precursor synthesis and 10 in their transport^[55], with studies showing that AtMS188 and OsMS188 regulate multiple genes in this pathway^[55,56]. For WT vs. *clms188*, the expression of 11 homologous genes in the sporopollenin synthesis and transport pathway was altered, including *CIACLB1*, *CIFAS*, *CIMS2*, *CICYP704B1*, *CIPKSA*, *CITKPR1*, *CIDPW2*, and *CILAP3*, and the sporopollenin precursor transporter family *CIABCG1*, *CIABCG9*, *CIABCG26* (Supplementary Fig. S11; Supplementary Table S10). This indicates that CIMS188 plays a critical role in the process of sporopollenin synthesis and transport. Interestingly, for WT vs. *clms111*, the expression of four genes in this pathway—*CICYP704B1*, *CITKPR2*, *CIDPW2*, and *CIABCG9*—also changed, suggesting that these genes may exhibit functional diversity (Supplementary Fig. S11; Supplementary Table S10). Additionally, KEGG analysis demonstrated the enrichment of 12 lipid metabolism pathways in both WT vs. *clms188* and WT vs. *clms111* comparisons, among which the steroid biosynthesis (ko00100) and cutin, suberine, and wax biosynthesis (ko00073) pathways are critical for pollen coat formation (Supplementary Table S9). We found that DEGs in the steroid biosynthesis pathway

only change in the comparison of WT vs. *clms111*, such as *CISMT2*, *CICAS1*, and *CISLM* (Supplementary Table S9). Notably, compared to the transcriptome data of the *clms111* mutant and microarray expression data of the *atms1* mutant^[34], both *CISLM* and *AtSLM* were found to be differentially expressed, respectively, suggesting that *CISLM* and *AtSLM* may share similar regulatory patterns. However, no differential expression of *AtSMT2* and *AtCAS1* was detected in the *atms1* mutant of *Arabidopsis*, indicating that these two genes may have undergone functional divergence between *Arabidopsis* and watermelon (Supplementary Table S9). Furthermore, in the cutin, suberine, and wax biosynthesis pathway, the number of DEGs in the WT vs. *clms111* was nearly double that in the WT vs. *clms188*, which means that CIMS1L1 plays an important role in pollen coat formation.

Based on the reported information about male sterility-related genes in *Arabidopsis*, rice, and maize^[17,57,58], we identified 25 homologous male sterility-related genes in WT vs. *clms188* (22 downregulated and three upregulated) and 16 homologous male sterility-related genes in WT vs. *clms111* (nine downregulated and seven upregulated) (gene names and Gene IDs see Table 1). For WT vs. *clms188*, the *CIDYT1-CITDF1-CIAMS-CIMS188-CIMS1L1* genetic pathway, genes *CIDYT1* and *CITDF1* were downregulated, while that of *CIAMS* was upregulated, suggesting a possible feedback regulatory mechanism between CIMS188 and *CIAMS*. As expected, *CIMS1L1* expression was undetectable, which further validates that *CIMS1L1*

acts downstream of CIMS188 and is directly regulated by CIMS188 (Fig. 3; Supplementary Table S5). In the sporopollenin-related pathway, the expressions of sporopollenin precursor synthesis- and transport-related genes *CIACLB1*, *CIMS2*, *CICYP704B1*, *CIPKSA*, *CITKPR1*, *CIDPW2*, *CILAP3*, *CIABCG1*, *CIABCG9*, and *CIABCG26* were altered (Table 1). In addition, the expressions of the lipid transfer protein genes *CIC6* and *CIMS44* were upregulated. This indicates that CIMS188 may be involved in the synthesis and transport of sporopollenin and lipid precursors. Recent studies showed that AtMS188 regulates AtQRT3 expression for tetrad wall pectin degradation^[28], and together with AtTEK co-regulates AtA6 and its homologs (AtA6s) expression for tetrad wall callose degradation^[59]. We found that the expression of *CIA6.2*, *CIA6.5*, and *CIQRT3* was downregulated in the *clms188* mutant. Additionally, the expression of *CICaIS5*, which regulates the deposition of callose in the tetrad wall, was downregulated. Therefore, CIMS188 may play an important role in anther tetrad wall development. Moreover, the expressions of *CIKCS7* and *CIKCS15*, involved in the synthesis of pollen coat lipids;

CIGPAT3, involved in anther cuticle biosynthesis; *CIUCL23*, involved in the flavonoid metabolism pathway; and *CIJAR1*, involved in the jasmonic acid biosynthesis pathway, were also downregulated (Table 1). Overall, CIMS188 primarily functions in sporopollenin biosynthesis and tetrad wall development.

For WT vs. *clms111*, the *CIDYT1-CITDF1-CIAMS-CIMS188-CIMS1L1* genetic pathway gene *CIDYT1* was upregulated. Additionally, the expression of a *bHLH* transcription factor family member, *CIATM1* (which regulates early tapetum development), and an *LBD* transcription factor family member, *CILBD10* (which regulates male gametophyte development), were downregulated. Pollen coat formation involves pollen coat precursor biosynthesis and transport, which share common biosynthetic pathways with sporopollenin precursor biosynthesis and transport^[60,61]. The expressions of sporopollenin precursor synthesis- and transport-related genes *CICYP704B1*, *CITKPR2*, *CIDPW2*, and *CIABCG9* were upregulated. Moreover, the expression of lipid synthesis gene *CIGPAT3* and lipid transport genes

Table 1. Identification of the DEGs genes associated with a potential male fertility phenotype.

Gene name	Gene ID	Log ₂ FC	Function of homologs protein function	Fertility of homologs gene male sterility (MS)
WT vs. <i>clms188</i>				
<i>CIDYT1</i>	<i>Cla97C05G107520</i>	-1.69	Early tapetum development	Complete MS
<i>CITDF1</i>	<i>Cla97C06G126110</i>	-2.08	Early tapetum development	Complete MS
<i>CIAMS1</i>	<i>Cla97C02G030840</i>	2.016	Early tapetum development	Complete MS
<i>CIMS1L1</i>	<i>Cla97C11G207480</i>	-5.98	Tapetum PCD, exine and pollen coat formation	Complete MS
<i>CIMS2</i>	<i>Cla97C04G069450</i>	-6.31	Sporopollenin synthesis	Complete MS
<i>CICYP704B1</i>	<i>Cla97C11G214660</i>	2.29	Sporopollenin synthesis	Complete MS
<i>CIPKSA</i>	<i>Cla97C05G080280</i>	-5.73	Sporopollenin synthesis	Partial MS
<i>CITKPR1</i>	<i>Cla97C02G036530</i>	-4.77	Sporopollenin synthesis	Partial MS
<i>CIDPW2</i>	<i>Cla97C06G119670</i>	-1.69	Sporopollenin synthesis	Complete MS
<i>CILAP3</i>	<i>Cla97C05G088990</i>	-12.39	Sporopollenin synthesis	Complete MS
<i>CIACLB1</i>	<i>Cla97C08G152670</i>	-1.13	Sporopollenin synthesis	Thermo-sensitive MS
<i>CIABCG1</i>	<i>Cla97C09G181690</i>	-4.85	Sporopollenin transportation	Redundant MS
<i>CIABCG9</i>	<i>Cla97C10G190610</i>	-4.65	Sporopollenin transportation	Complete MS
<i>CIABCG26</i>	<i>Cla97C10G188910</i>	-5.69	Sporopollenin transportation	Complete MS
<i>CIC6</i>	<i>Cla97C05G094260</i>	-2.57	Lipid transportation	Partial MS
<i>CIMS44</i>	<i>Cla97C05G089860</i>	1.05	Lipid transportation	Complete MS
<i>CIA6.2</i>	<i>Cla97C03G066330</i>	-1.26	Callose dissolution	Redundant MS
<i>CIA6.5</i>	<i>Cla97C11G209110</i>	-1.01	Callose dissolution	Redundant MS
<i>CIQRT3</i>	<i>Cla97C01G025690</i>	-1.14	Pectin dissolution	Complete MS
<i>CICaIS5</i>	<i>Cla97C02G026770</i>	-2.90	Callose biosynthesis	Partial MS
<i>CIKCS7</i>	<i>Cla97C10G196270</i>	-2.66	Pollen coat synthesis	Partial MS
<i>CIKCS15</i>	<i>Cla97C05G096910</i>	-1.19	Pollen coat synthesis	Partial MS
<i>CIGPAT3</i>	<i>Cla97C09G183960</i>	-3.10	Anther cutin biosynthesis	Complete MS
<i>CIUCL23</i>	<i>Cla97C11G213980</i>	-1.15	Flavonoid metabolism	Partial MS
<i>CIJAR1</i>	<i>Cla97C05G081210</i>	-1.49	JA biosynthesis	Complete MS
WT vs. <i>clms111</i>				
<i>CIDYT1</i>	<i>Cla97C05G107520</i>	2.25	Early tapetum development	Complete MS
<i>CIATM1</i>	<i>Cla97C06G117840</i>	-1.09	Tapetum PCD	Complete MS
<i>CILBD10</i>	<i>Cla97C02G038990</i>	-2.27	Pollen development	Partial MS
<i>CICYP704B1</i>	<i>Cla97C11G214660</i>	1.93	Sporopollenin synthesis	Complete MS
<i>CITKPR2</i>	<i>Cla97C05G104620</i>	1.80	Sporopollenin synthesis	Partial MS
<i>CIDPW2</i>	<i>Cla97C06G119670</i>	2.37	Sporopollenin synthesis	Complete MS
<i>CIABCG9</i>	<i>Cla97C10G190610</i>	-2.86	Sporopollenin transportation	Complete MS
<i>CIGPAT3</i>	<i>Cla97C09G183960</i>	2.25	Lipid metabolism	Complete MS
<i>CIMS44</i>	<i>Cla97C05G089860</i>	-2.32	Lipid transportation	Complete MS
<i>CIC6</i>	<i>Cla97C05G094260</i>	-1.13	Lipid transportation	Partial MS
<i>CILecRK5</i>	<i>Cla97C08G149390</i>	1.43	Callose biosynthesis	Complete MS
<i>CIG1</i>	<i>Cla97C03G061520</i>	2.00	Callose degradation	Complete MS
<i>CIKCS5</i>	<i>Cla97C05G089490</i>	-5.12	Pollen coat synthesis	Partial MS
<i>CIKCS7</i>	<i>Cla97C10G196270</i>	-1.02	Pollen coat synthesis	Partial MS
<i>CIGELP34</i>	<i>Cla97C07G138280</i>	-1.44	Pollen exine formation	Complete MS
<i>CIUAM3</i>	<i>Cla97C06G125020</i>	-1.71	Pollen cell wall formation	Partial MS

Further details and references for these genes are provided in Supplementary Table S12.

CIMS44 and *CIC6* were altered. *AtKCS* family genes *AtKCS7*, *AtKCS15*, and *AtKCS21* were reported to be downstream of *AtMS1* and are involved in the accumulation of pollen coat lipids^[35]; the expressions of *CIKCS5* and *CIKCS7* were downregulated. Furthermore, the expressions of the pollen exine formation-related gene *CIGELP34*, the pollen wall formation-related gene *CIUAM3*, the callose synthesis gene *CILeCRK5*, and the callose degradation gene *CIG1* were upregulated (Table 1). These results suggest that *CIMS1L1* plays a key role in regulating the lipid metabolism essential for pollen coat formation.

Notably, in the *Arabidopsis atms1* mutant background, pollen coat-related genes such as the *GRP* (glycine-rich protein) family genes (*GRP14*, *GRP16*, *GRP17*, *GRP18*, *GRP20*) and the *EXL* (extracellular lipase protein) family genes (*EXL4*, *EXL5*, *EXL6*) were altered^[32], both of which are involved in pollen hydration. However, in the *clms11* mutant, we did not detect changes in the corresponding homologous genes, suggesting that the genes related to pollen hydration in watermelon may have diverged from those in *Arabidopsis* (Supplementary Table S9).

Discussion

Identification of the *CIDYT1-CITDF1-CIAMS-CIMS188-CIMS1L1* genetic pathway in watermelon anther development

Although the genetic pathway composed of these five transcription factors that regulates anther development has been identified in *Arabidopsis* (*AtDYT1-AtTDF1-AtAMS-AtMS188-AtMS1*), rice (*OsUDT1-OsTDF1-OsTDR-OsMS188-OsPTC1*), and maize (*ZmMs32-ZmMs9-ZmHLH51-ZmMYB84-ZmMs7*)^[17,21–23] (see Supplementary Table S13 for details), molecular mechanisms regulating tapetal development and pollen maturation in watermelon anthers are still poorly understood. Through genetic and molecular analyses, we validated the functions of the downstream genes of the *CIDYT1-CITDF1-CIAMS-CIMS188-CIMS1L1* genetic pathway, *CIMS188* and *CIMS1L1*, to study the regulatory relationship. The results demonstrate that *CIMS188* directly binds to the TAACCA motif of *CIMS1L1* and activates its

transcription (Fig. 3). By analyzing the transcriptomic regulatory network of *CIMS188* and *CIMS1L1*, the results further confirm the existence of the complete *CIDYT1-CITDF1-CIAMS-CIMS188-CIMS1L1* genetic pathway in watermelon anther development, which precisely regulates the development of the tapetum and pollen in watermelon (Fig. 7).

CIMS188 plays a crucial role in tetrad wall degradation and sporopollenin precursor formation

CIMS188 plays a central role in watermelon anther tapetal development and pollen formation. As a homolog of *AtMS188/OsMS188*, its expression peaks during the tetrad stage (stage 8b) (Fig. 1b, c). Similar to its function in model plants, transcriptomic analysis revealed that *CIMS188* coordinates multiple critical processes in anther development by regulating a set of key genes. This includes controlling genes involved in tapetal development (e.g., *CIDYT1*, *CITDF1*, and *CIAMS*), tetrad wall degradation (e.g., *CIQRT3* and *CIA6s*), and genes regulating sporopollenin biosynthesis and transport (e.g., *CIMS2* and the members of the *CIABCG* gene family) (Table 1). These regulatory functions are essential for both tetrad wall degradation and sporopollenin precursor formation (Fig. 7). Furthermore, our experimental results indicate that *CIMS188* directly binds to the TAACCA motif, a specific *cis*-acting element in the *CIMS1L1* promoter, and activates its transcription, thereby promoting pollen coat formation. Analysis of the phenylpropanoid biosynthesis pathway indicated that *CIMS188* regulates genes involved in anther wall lignification (e.g., *CICCR* and *CI CAD*) (Fig. 6). This is supported by the more pronounced downregulation of these lignin-related genes in the *clms188* mutant compared to the *clms11* mutant. This mechanism allows *CIMS188* to precisely coordinate the mechanical strengthening of the anther wall (via lignin) and the metabolic maturation of pollen grains (via *CIMS1L1*).

CIMS1L1 is involved in regulating pollen coat formation

The orthologs *AtMS1/OsPTC1* are highly expressed at the microspore release stage (stage 9), regulating tapetal PCD, pollen exine, and pollen coat formation.

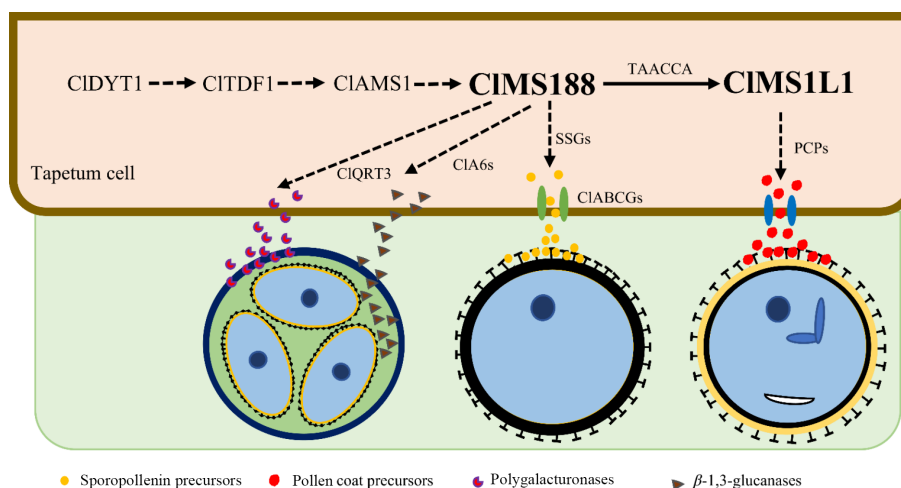


Fig. 7 A proposed model for the regulation of five transcription factors during anther development in watermelon. *CIMS188* and *CIMS1L1* are downstream components in the *CIDYT1-CITDF1-CIAMS-CIMS188-CIMS1L1* genetic pathway. *CIMS188* directly activates *CIMS1L1* expression; *CIMS188* may control the expression of *CIQRT3*, *CIA6s*, and sporopollenin biosynthesis and transport genes. *CIMS1L1* may control pollen coat protein formation. The solid arrow represents a validated regulatory mechanism, and the dashed arrows represent predicted regulatory mechanisms. SSGs: sporopollenin biosynthesis genes; PCPs: pollen coat precursors.

and coat formation^[25,32,34,62,63]. Similarly, *CIMS1L1* expression peaks at stage 9 (Fig. 4a, b). The *clms1l1* mutant exhibits aberrant tapetum and microspore development from this stage (Fig. 4i), confirming its crucial role. Transcriptome data show that *CIMS1L1* downregulates genes for pollen coat lipids (e.g., *CIKCS5/7*), pollen exine formation (*CIGELP34*), and sporopollenin biosynthesis/transport (e.g., *CICYP704B1*, *CITKPR2*, *CIDPW2*, and *CIABCG9*). Genes involved in lipid synthesis/transport (e.g., *CIGPAT3*, *CIMS44*, *CIC6*) were altered. This likely impairs lipid metabolism, reducing pollen adhesiveness and affecting insect pollination. Thus, *CIMS1L1* primarily regulates the synthesis and transport of pollen coat precursors and lipids during maturation. The altered Auxin/JA hormone signaling is a metabolic consequence, not a primary cause. Disrupting the phenylpropanoid pathway in mutants blocks the production of sporopollenin and lignin, causing upstream phenolic precursors to accumulate. These intermediates can inhibit/regulate auxin transport and JA biosynthesis; therefore, the hormonal imbalance is a feedback response to the blocked metabolic flux (Figs. 5, 6). This metabolic-hormonal crosstalk synchronizes anther dehiscence with pollen maturation.

***CIMS188* and *CIMS1L1* can be applied to the SPT system in watermelon**

The *clms188* and *clms1l1* mutants are recessive genic male sterility (RGMS) mutants and exhibit no developmental defects in traits including vegetative growth and female reproductive organ development (Supplementary Figs. S3, S6; Supplementary Table S11). Crucially, the *clms188* and *clms1l1* mutants exhibit a 'clean' male sterility phenotype without pleiotropic defects (Figs. 2, 4). Unlike some male-sterile mutants in other crops that suffer from vegetative growth inhibition or abnormal female fertility, these watermelon mutants maintain normal vegetative vigor and female fruit set. This specificity renders them ideal GMS resources for hybrid seed production. Furthermore, studies on the *MS1* homolog in cabbage have demonstrated that a natural allele, *Ms-cd1_{PA-597}*, resulting from a 1-bp deletion in its promoter, confers dominant genic male sterility (DGMS). We propose to use the ABE/GBE technologies developed in our laboratory to precisely mutate the corresponding site in the promoter of *CIMS1L1*, thereby potentially generating DGMS lines that can serve as a genetic resource for the SPT system^[64,65]. They can be utilized to eliminate the labor-intensive manual emasculatation process in watermelon breeding, significantly reducing costs while ensuring hybrid purity^[7,66].

Conclusions

In this study, loss-of-function mutations in *CIMS188* and *CIMS1L1* resulted in complete male sterility. Expression analyses and anther sectioning demonstrated that *CIMS188* and *CIMS1L1* are specifically expressed in anthers at stage 8b and stage 9, during which abnormal anther development phenotypes were first observed. LUC, Y1H, and EMSA assays confirmed that *CIMS188* directly activates *CIMS1L1* transcription. Comparative transcriptomic analyses revealed that *CIMS188* coordinates anther development through two separate pathways: one independent of *CIMS1L1*, which regulates tetrad wall degradation and sporopollenin biosynthesis, and another dependent on *CIMS1L1*, which controls lipid metabolism for pollen coat formation.

Author contributions

The authors confirm their contributions to the paper as follows: conceived and designed the study: Yuan L; executed the

experiments: Zhang Q, You X, Peng Y, Zhang Y, Yang F, Zhong Y; provided experimental technical support: Chen X; conducted data analysis: Zhang Q; provided project supervision: Yuan L, Tian S, Luan F; wrote the manuscript: Yuan L, Zhang Q. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The RNA-Seq data were deposited in the NCBI Sequence Read Archive (SRA) database under the accession PRJNA1496536 (<https://dataview.ncbi.nlm.nih.gov/object/PRJNA1496536>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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