The SlHB8 acts as a negative regulator in tapetum development and pollen wall formation in Tomato

Caiyu Wu1,†, Yang Yang1,†, Deding Su2,3,†, Canye Yu1, Zhiqiang Xian2,3, Zanlin Pan1, Hongling Guan1, Guojian Hu4, Da Chen1, Zhengguo Li2,*, Riyuan Chen1,† and Yanwei Hao1,†

1Key Laboratory of Horticultural Crop Biology and Germplasm Innovation in South China, Ministry of Agriculture, College of Horticulture, South China Agricultural University, Guangzhou 510642, China
2Key Laboratory of Plant Hormones and Development Regulation of Chongqing, School of Life Sciences, Chongqing University, Chongqing, China
3Center of Plant Functional Genomics, Institute of Advanced Interdisciplinary Studies, Chongqing University, Chongqing 400044, China
4UMR990 INRA/INP-ENSAT, Université de Toulouse, Castanet-Tolosan, France

†Corresponding authors. E-mails: yanweihao@scau.edu.cn; zhengguoli@cqu.edu.cn; rychen@scau.edu.cn

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Article

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Introduction

Pollen development is crucial for the fruit setting process of tomatoes, but the underlying regulatory mechanism remains to be elucidated. Here, we report the isolation of one HD-Zip III family transcription factor, SlHB8, whose expression levels decreased as pollen development progressed. SlHB8 knockout using CRISPR/Cas9 increased pollen activity, subsequently inducing fruit setting, whereas overexpression displayed opposite phenotypes. Overexpression lines under control of the 35 s and p2A11 promoters revealed that SlHB8 reduced pollen activity by affecting early pollen development. Transmission electron microscopy and TUNEL analyses showed that SlHB8 accelerated tapetum degradation, leading to collapsed and infertile pollen without an intine and an abnormal exine. RNA-seq analysis of tomato anthers at the tetrad stage showed that SlHB8 positively regulates SPL/NZZ expression and the tapetum programmed cell death conserved genetic pathway DYT1–TDF1–AMS–MYB80 as well as other genes related to tapetum and pollen wall development. In addition, DNA affinity purification sequencing, electrophoretic mobility shift assay, yeast one-hybrid assay and dual-luciferase assay revealed SlHB8 directly activated the expression of genes related to pollen wall development. The study findings demonstrate that SlHB8 is involved in tapetum development and degradation and plays an important role in anther development.
the conserved PCD genetic pathway was predicted to be present in tomato, other regulators involved in this process remain to be clarified.

The class III homeodomain-leucine zipper (HD-Zip III) transcription factor family were reported to determine the ad/abaxial polarity of leaves, anthers, vascular organs, and developing embryos [13,33–37]. Five HD-Zip III genes including FHBULOSA (FHB)/ATHB14, REVOLUTA (REV), PHAVOLUTA (FHV)/ATHB9, INCURVATA4/CORONA/ATHB15, and ATHB8 were identified in the Arabidopsis genome [38]. These HD-Zip III genes show overlapping expression and exhibit redundant functions. Single loss-of-function of these genes gives non-obvious phenotypes. Simultaneous mutation of REV, PHV, and PHB affected meristem formation and seedlings structure [38, 39]. HD-Zip III mRNA can be degraded by the microRNA miR165/6 [40]. Up-regulation of HD-Zip III genes via disruption of miRNA regulation sites result in strong developmental phenotypes [37, 41, 42]. Gain-of-function of PHB and PHV displayed leaves with damaged polarity and abnormal appearance [41]. MiR166-PHB-SPL/NZZ of-function of PHB and PHV displayed leaves with damaged polarity and abnormal appearance [41]. MiR166-PHB-SPL/NZZ module regulates the stamen polarity through modifying the boundary thickness [13]. In cucumber, CsSPL formed a complex with CsPHB and CsWUS to orchestrate sex organ development in an unidentified regulatory pathway [12]. There are six HD-Zip III genes in tomato, including Slolc11g069470, Slolc12y044410, Slolc03y120910 (SlHB15A), Slolc02g024070, and Slolc02g069830 [43]. These genes are negatively regulated by miR166, overexpression of Sly-pre-miR166b down-regulates all six HD-Zip III genes, and the plant bears a fruit with another fruit developing inside or parthenocarpic fruit [44]. Overexpression of SIREV does not result in a discernable plant phenotype, but its overexpression via disruption of microRNA regulation sites leads to ectopic flower formation and fused fruit [44]. SlHB15A regulates parthenocarpic fruit set under cold conditions via miR166-mediated recessive dosage sensitivity [43]. Although the effect of SIREV and SlHB15A genes on fruit development has been clarified, its role in anthers need to be investigated.

Here, we isolated one HD-Zip III family transcription factor, SlHB8, whose loss-of-function increased pollen activity, inducing fruit setting. SlHB8 overexpression showed the opposite phenotype. In order to clarify the function of SlHB8 in pollen development, cytobiology and molecular biology technologies were carried out on the anthers at different development stages. The results proved SlHB8 was a negative regulator during pollen development by mediating the tapetum development through conserved genetic pathway DYT1–TDF1–AMS–MYB80.

Results

**SlHB8 shows a pollination-dependent expression pattern**

The SlHB8 transcription factor belongs to the HD-ZIP III transcription factor family, as it contains the four conserved domains: HD, bZIP, START, and PAS [45]. SlHB8 is expressed in all tissues, such as the root, stem, leaves, flowers, mature green fruits, breaker fruits, and red fruits, showing highest expression level in stem and lowest expression level in the red rip fruit (Fig. S1a, see online supplementary material). The highest expression level in the stem is in line with the enlarged stem diameter of SlHB8 knocking out lines [46]. Here, SlHB8 was detected in the petal, sepal, stamen, and carpel flower organs and showed the highest expression level in sepalas, as evidenced by qPCR (Fig. S1b, see online supplementary material). During stamen development, SlHB8 expression decreased (Fig. S1c, see online supplementary material). The in situ hybridization result showed that SlHB8 transcripts were found in the microspore and tapetum cells from microspore mother cell stage to the mature pollen stage (Fig. 1), indicating its role in the pollen development. SlHB8 is induced after treatment with auxin, gibberellic acid, and artificial pollination [45]. When the fruit set succeeded, SlHB8 expression levels increased when compared to ovaries before pollination (Fig. S1d, see online supplementary material). These data suggest that SlHB8 is important for stamen development and the fruit setting process.

**Knocking out SlHB8 via CRISPR/Cas9 promotes fruit set rate and pollen activity in tomato**

To determine its role in anther development and the fruit setting process, we knocked out SlHB8 using CRISPR/Cas9; SlHB8-specific primers and sequencing were used to verify the knockout effect. Three mutant types were obtained, including two with an 8 bp deletion in the CDS and one with a 1 bp insertion (Fig. 2a). Phenotyping was performed for the two deletion lines. Compared with the WT, the fruit set rates were higher in the SlHB8 knockout lines (Fig. 2b), but the fruit size, weight, and seed number (Fig. 2c and d Fig. S2a and b, see online supplementary material) did not change. Pollen activity, pollen tube length, and the pollen tube germination rate were higher in the SlHB8 knockout lines than that in the WT (Fig. 3a,b–d, Fig. S2c–e, see online supplementary material), and the anther width was thinner (Fig. 5f, see online supplementary material).

**Overexpression of SlHB8 results in pollen abortion and seedless fruits**

An miRNA166 target site was found in the SlHB8 gene, and thus individual SlHB8 overexpression lines under control of the 35S and fruit-specific 2A11 promoters were generated by mutating the miRNA166 target site [47]; transgenic plants were verified by qPCR, with seven p35S:SlHB8Ris and seven p2A11:SlHB8Ris lines showing overexpressed SlHB8 levels (Fig. 2e). Most p35S:SlHB8Ris overexpression lines produced seedless fruits (Fig. 2d), therefore, only two lines (line 1 and 2) were collected from a few seeds [47]. Compared with the WT, the p35S:SlHB8Ris overexpression transgenic plants bore fewer fruits (Fig. 2f) and the fruit size, weight, and seed number (Fig. 2g, Fig. S3a and b, see online supplementary material) were significantly reduced. All p2A11:SlHB8Ris overexpression lines displayed phenotypes – such as fruit set, seed number, fruit size, and fruit weight – similar to those of the WT tomato plants (Fig 2f and g, Fig. S3a and b, see online supplementary material).

Because pollen viability and ovule development affects the fruit set rate and seed number, which were reduced in the L1 and L2 lines, reciprocal cross experiments between p35S:SlHB8Ris and the WT as well as p2A11:SlHB8Ris and the WT were performed. When the WT was used as the female parent and p35S:SlHB8Ris as the male parent, the fruit set rate reached 2.7%, however, the fruit set rate decreased to 93.5% when this was switched (Table 1). In contrast to p35S:SlHB8Ris, the fruit set rate remained similar regardless of the pollen donor when WT and p2A11:SlHB8Ris were crossed (Table 1). These results indicate that the pollen varieties were mainly responsible for the lower fruit set rate and reduced seed number. We thus assessed the viability of mature pollen grains at the anthesis stage using the TTC method. Via microscopy, pollen grains of p35S:SlHB8Ris were found defective, whereas those of p2A11:SlHB8Ris and SlHB8-cr were similar to those of the WT (Fig. 3a and b). In addition to pollen viability, pollen shape, pollen tube length, and pollen tube
Figure 1. RNA in situ hybridization of SIHB8 during anther development of wild-type tomato plant. Anthers at MI, Tds, MUM, BM and MP stages were cross-sectioned for hybridization with antisense (upper) and sense (lower) probes of SIHB8. Black and white arrows indicate positive and negative in situ hybridization signals for SIHB8 transcripts respectively. BM: binucleate microspore stage; MI: microspore mother cell stage; MP: mature pollen stage; MSP: microspore pollen; MUM: middle uninucleate microspore stage; PG: pollen grain; T: tapetum; Td: tetrad; Tds: tetrad stage. Bars = 50 μm.

Figure 2. Phenotyping of SIHB8 gene overexpression and knockout plants. a Three SIHB8 gene knockout lines were established using CRISPR/Cas9. Fruit set rate (b) and the number of seeds per fruit (c) of SIHB8 knockout plants. d Photos of cross-section of red ripe fruits of wild-type, SIHB8 gene knockout and SIHB8 overexpression lines. Scale bar = 1 cm. e Expression levels of SIHB8 under control of the 35 s and 2A11 promoters in the SIHB8 overexpression lines. Ubi was used as a reference gene. Fruit set rate (f) and the number of seeds per fruit (g) of SIHB8 gene overexpression plants. The error bars denote SE; ∗P < 0.5, **P < 0.01, ***P < 0.001 (Student’s t-test; compared with the WT). 35 s-L1, promoter 35 s-driven SIHB8 overexpression line 1; 2A11-L17, promoter 2A11-driven SIHB8 overexpression line 17.

germination capacity showed observable differences between p35s::SIHB8Ris and the WT (Fig. 3a–c, Fig. S3c–e, see online supplementary material), and the p35s::SIHB8Ris anther width was thinner (Fig. S3f, see online supplementary material). By contrast, pollen viability, pollen tube length, pollen tube germination capacity, and morphology of the p2A11::SIHB8Ris lines were similar to those of the WT (Fig. 3a–c, Fig. S3c–e, see online supplementary material). SEM was then employed to observe the whole structure of mature pollens at the anthesis stage. Unlike the round and regularly shaped WT pollen grains, the p35s::SIHB8Ris transgenic pollen grains were irregular, shrunken, and collapsed. The pollen grain surface was also different from that of the WT (Fig. 3a). No significant differences were observed between p2A11::SIHB8Ris, SIHB8cr, and the WT (Fig. 3a). Next, transmission electron microscopy (TEM) was used to investigate the ultrastructural changes of the aborted p35s::SIHB8Ris pollen grains; the exine layer of irregular pollen grains was wider in p35s::SIHB8Ris than in the WT, and the intine layer between the plasma membrane and exine was absent in the p35s::SIHB8Ris lines (Fig. 4).

Because of the different effects of SIHB8 on fruit morphology in the p35s::SIHB8Ris and p2A11::SIHB8Ris overexpression lines, we compared SIHB8 expression levels during anther development. The SIHB8 gene was overexpressed throughout anther develop-
Figure 3. Pollen viability and pollen morphology of \( \text{SHHB8} \) gene knockout and overexpression lines. **a** Scanning electron micrographs of pollen grains from wild-type, \( \text{SHHB8} \) gene knockout and \( \text{SHHB8} \) overexpression lines under control of the 35 s and 2A11 promoters. BM: binucleate microspore stage; MI: microspore mother cell stage; MP: mature pollen stage; MUM: middle uninucleate microspore; Tds: tetrad stage. From the top to the bottom, the scale bar indicated 20 \( \mu \)m, 2 \( \mu \)m, 1 \( \mu \)m, respectively. **b** Pollen viability after TTC staining. Scale bar=100 \( \mu \)m. **c**, **d** Percentage of pollen viability in wild-type and \( \text{SHHB8} \) transgenic lines. **e**, **f** Expression levels of \( \text{SHHB8} \) in the transgenic tomato lines \( \text{p35s::SlHB8Ris} \) and \( \text{p2A11::SlHB8Ris} \) during anther development. \( \text{Ubi} \) was used as reference gene. Expression level in MI stage flower bud was used as control. The error bars denote SE; **∗∗∗** \( P < 0.001 \) (Student’s \( t \)-test; compared with the WT).

Table 1. Cross-fertilization assay.

|                   | \( \text{SHHB8-ox♂} \times \text{WT♀} \) | \( \text{WT♂} \times \text{SHHB8-ox♀} \) | \( \text{2A11-SHHB8♂} \times \text{WT♀} \) | \( \text{WT♂} \times \text{2A11-SHHB8♀} \) |
|-------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Fruit set         | 2.70%                           | 93.50%                          | 60.60%                          | 50%                             |
| Hybrid number     | 37                              | 31                              | 33                              | 30                              |
| Setted fruits number | 1                               | 29                              | 20                              | 15                              |

emasculated wild type flowers were fertilized with \( \text{SHHB8-ox} \) pollen and the number of setting fruit [lr1] was assessed at the ripe stage. Conversely, tomato pollen from wild type flowers was used to fertilize emasculated \( \text{SHHB8-ox} \) flowers. The same assay was also carried out on the 2A11 lines.

Overexpression of \( \text{SHHB8} \) disrupts pollen development due to early tapetal PCD

To identify the key stage at which \( \text{SHHB8} \) regulates pollen development, we defined the pollen development stages as MI, Tds, MUM, BM, and MP (anthesis) using DAPI staining (Fig. 5). At the MI and Tds, the nuclei and tetrad formed normally both in the WT, \( \text{SHHB8cr} \) and \( \text{p35s::SHHB8Ris} \) transgenic lines. Starting from the MUM stage to the MP stage, the nucleus disappeared in the \( \text{p35s::SHHB8Ris} \) lines and the pollen shape became irregular and collapsed, which is obviously different from that of WT and \( \text{SHHB8cr} \) anthers with two nuclei and round, flush pollen grains (Fig. 5a). We further performed a set of cytological experiments to characterize the spatial and temporal cytological defects in \( \text{p35s::SHHB8Ris} \) anthers. In agreement with the above observations, histological anther sections showed that at the MI stage, the cell layer differentiation appeared similar to that of WT anthers, and a tetrad formed in both the WT, \( \text{SHHB8cr} \) and \( \text{p35s::SHHB8Ris} \) anthers during the Tds; there were no observable defects in the anthers during these two stages (Fig. 5b, Fig. S4, see online supplementary material). At the MUM stage, abnormal pollen grains with an irregular shape and vacuolation appeared in the \( \text{p35s::SHHB8Ris} \) anthers, and most of the pollen grains were aborted in the \( \text{p35s::SHHB8Ris} \) lines (Fig. 5b). Via TEM, we observed a large nucleus in the WT and \( \text{SHHB8cr} \), which was not present in the \( \text{p35s::SHHB8Ris} \) lines (Fig. 4). Instead, there was an increased number of large vacuoles, and the intine of the pollen wall was absent during the MUM stage (Fig. 4). During the WT and \( \text{SHHB8cr} \) BM stage, the microspore contained a full cytoplasm with normal vegetative and generative nuclei (Fig. 4). However, the majority of the cytoplasm and nuclei in pollen grains of the \( \text{p35s::SHHB8Ris} \) lines were completely degraded, and only trace cytoplasmic inclusions were observed (Fig. 4). At the Tds, the \( \text{p35s::SHHB8Ris} \) anther tapetum was thinner than that of the WT and \( \text{SHHB8cr} \), and tapetal cells were infertile and shrunken (Fig. 6a).
Tapetum PCD is one of the key factors affecting tapetal cell degradation and pollen development. To assess whether the infertile tapetal cells are associated with early PCD, we performed a TUNEL assay, which provides strong fluorescent signals when cells undergo massive DNA fragmentation. In the WT and SlHB8cr-1, a positive signal appeared at the 4 mm anther stage (Tds), which was observed earlier in p35s::SlHB8Ris at the 3 mm anther stage (Fig. 6b). The results indicate that SlHB8 overexpression accelerates tapetal cell degradation, leading to pollen abortion.

**Primary metabolite determination in p35s::SlHB8Ris anthers at the mature pollen stage**

Given that anther development is associated with metabolite levels, we determined primary metabolite content in the WT and p35s::SlHB8Ris anthers. Primary metabolites measured included amino acids and derivatives; nucleotides and derivatives; carbohydrates; indole derivatives; organic acids and derivatives; and lipids. All nucleotides and derivatives were down-regulated, and with the exception of O-rhamnoside, levels of the other three carbohydrates measured were reduced. All lipids detected were significantly up-regulated, as well as half of the organic acids and derivatives, with the other half down-regulated. All indole derivatives were down-regulated in the p35s::SlHB8Ris anthers (Fig. S5; Table S1, see online supplementary material). The correlation among the three biological replicates was qualified by Pearson’s correlation coefficient ($R^2$), with $R^2 > 0.8$ as the significance cutoff. The $R^2$ value of the three biological replicates was >0.99, indicating a high correlation (Fig. S6, see online supplementary material).

To identify DEGs in tetrad stage anthers between the WT and p35s::SlHB8Ris lines, pairwise comparisons were performed with |log2 (fold change)| > 1 and FDR < 0.05 as cutoff thresholds to filter the significant DEGs. These comparisons allowed the identification of 900 DEGs, including 355 down-regulated and 545 up-regulated genes (Fig. 7a; Table S3, see online supplementary material). Among these 900 DEGs, genes involved in the regulation of microspore protein biosynthesis, tapetum development, callose metabolism, pollen inner wall formation, pollen outer wall formation, and hormone signal transduction pathways related to pollen development were up-regulated (Fig. 7d).

qRT-PCR was then performed using seven randomly selected genes to confirm the accuracy of RNA-seq. All seven genes exhibited similar expression pattern and high Pearson’s correlation coefficient [RNA-seq and qRT-PCR: 0.9715 (P < 0.0001)], indicating that the transcriptome data were highly reliable (Fig. S7, see online supplementary material).
As SlHB8 was predicted to be an activator, the up-regulated DEGs may represent a direct response of SlHB8 overexpression. To further understand the putative functions of these 545 up-regulated DEGs, gene ontology (GO) assignment and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were carried out. Using a significant cutoff value of q ≤ 0.05, the data revealed that the 545 DEGs were only significantly enriched in the biological process term related to pollen wall assembly, pollen development, and gametophyte development (Fig. 7c; Table S4, see online supplementary material), with six KEGG pathways significantly enriched (Fig. 7e; Table S4, see online supplementary material): 'phenylalanine metabolism', 'tyrosine metabolism', 'isoquinoline alkaloid biosynthesis', 'phenylpropanoid biosynthesis', 'pentose and glucuronate interconversions', and 'cutin, suberin and wax biosynthesis'. The last three pathways have been proved to be related to pollen development (Fig. 7e).

Identification of SlHB8-targeted DEGs by DAP-seq

DEGs identified by RNA-seq were directly or indirectly regulated by SlHB8. To identify DEGs directly regulated by SlHB8, we used DAP-seq to identify the SlHB8-binding sites in vitro. In total, 71,504,200 bp peaks were identified in the tomato genome, including 12,627 (17.66%) peaks that were presented within 2.0 kb upstream of the annotated ORFs, 4583 (6.41%) located within 300 bp downstream of putative ORFs, and the remaining 54,293 (75.93%) distributed in introns, exons, or intergenic regions in the genome (Fig. S8; Table S5, see online supplementary material). To confirm the DAP-seq results, we selected four binding elements for Y1H and EMSA assays. SlHB8 interacted with all tested elements (Fig. 8a and b). Furthermore, we performed a dual-luciferase assay using five genes (SlVPE, SlCPS2, SlLAT52, SlGH3.1, and SlMYB80) whose promoters contained the target motifs of SlHB8 and found that SlHB8 activated their expression except SlMYB80 which showed a slight up-regulation without significance (Fig. 8c). After analyzing the genes with promoters containing the DAP-seq fragments and the SlHB8-regulated DEGs, we revealed 150 overlapping genes (Fig. 7b; Table S6, see online supplementary material). Among these genes, there were genes involved in sporopollenin biosynthesis and transport, tapetum development, hormone metabolism and signaling pathways, pollen intine development, callose metabolism and genes belong to transcription...
factors, indicating that SlHB8 regulates pollen development by mediating these pathways (Fig. 7d; Table S6, see online supplementary material).

Discussion

SlHB8 regulates pollen development by disturbing tapetum PCD

The tapetum and its degradation triggered by PCD which produced enzymes, nutrients, and precursors play an important role in microspore and pollen wall development [3]. Numerous reports have shown that incorrect timing of tapetal PCD (premature or delayed degradation) and disintegration induces male sterility and pollen wall defects [3, 6–9, 16, 29]. In contrast to previous results and our findings in the WT, tapetum PCD in the SlHB8 overexpression lines occurred early in tomato anthers, before the tetrad stage, while tapetum PCD in the SlHB8 gene knockout lines appeared at the tetrad stage (Fig. 6b).

Over the past decade, the functionally conserved genetic pathway DYT1–TDF1–AMS–MYB80 in tapetum development and degradation has been identified in Arabidopsis, rice, tomato, and other crops [3, 7, 11, 17, 26]. Loss-of-function of these regulators results in aborted pollen and induces male sterility [6, 8–10, 16, 17, 26]. DYT1 is a critical transcription factor for early tapetum development and function upstream of TDF1, AMS, MYB80, TEK, and MS1 [16, 17]. TDF1 is the direct target gene of DYT1, and mutations in TDF1 severely impair tapetal development and callose dissolution. Tapetal cells in td1 fail to transit to the secretory type as a result of the low expression levels of MYB80 [9, 16]. AMS and MYB80 act as master regulators of pollen wall development due to its regulation on the genes related to callose degeneration (QRT3 and A6), genes related to sporopollenin biosynthesis and metabolism (CYP86C3, ACOS5, and SHT), genes related to lipid transport (LACS6 and WBC27), and pollen coat formation (EXL4–EXL6) [9, 16, 23]. Interestingly, our RNA-seq data showed that the core transcription factors that regulate tapetal PCD (SIDYT1, STTDF1, and SIMYB80) and their multiple downstream targets (e.g. SlA6, CYP703A2, CYP704B1, SlPKSA, and SlPKSB) were up-regulated in the SlHB8 overexpression lines (Fig. S7d); SlAMS was also significantly up-regulated (fold ~2) (Table S7, see online supplementary material). Moreover, DAP-seq, Y1H, and dual-luciferase assays confirmed that some genes were directly targeted by SlHB8, such as SIMYB80 and SlA6 (Fig. 8; Table S6, see online supplementary material), indicating that SlHB8 induces tapetum PCD by regulating these key tapetal pathway genes. Moreover, in the SlHB8 overexpression lines, TUNEL-positive signals were observed in the microspores before the tetrad stage (Fig. 6b), suggesting early callose generation and early secretory tapetal cell transition, which may induce early tapetum degradation. The overexpression of SIMYB80 and SlA6 also supports the hypothesis that early decretory tapetal cell transition leads to early tapetum PCD. While loss-of-function of SlHB8 has no effect on the tapetum PCD signal and expression of tapetum PCD regulators (Fig. 6b, Fig. S9c, see online supplementary material), indicating another gene may compensate the function of SlHB8 or another partner work together with SlHB8 in regulating the tapetum PCD.

SlHB8 may function upstream of SlSPL to regulate early pollen development

SPL has been reported to function in the process of sex organ development [12–14, 48, 49]. In Arabidopsis, SPL/NZZ controls early microsporocyte differentiation. Loss-of-function of SPL resulted in inhibited microsporocyte formation and deformed tapetum [13, 14]. In cucumber, CcSPL formed a complex with HD-Zip III and CsWUS to regulate anther and ovule development [12]. In tomato, the SlSPL loss-of-function mutant hydra showed sterility phenotypes both on male and female organs [49]. In developing anthers of Arabidopsis, miR165/6 acts as a regulator balancing the expression of PHB and SPL/NZZ to determine the polarity of the anthers [13]. In addition to PHB, the adaxial identity genes include the other HD-Zip III family genes (REV, PHB, PVH, CNA, and ATHB8), which are also repressed by microRNA165/6 [50]. Overexpression of SlHB8 with a mutated miR165 target site resulted in aborted pollen grains (Fig. 3a–c) and up-regulation of SlSPL (Table S3, see online supplementary material), indicating its upstream function during tomato anther development. According to the DAP-seq data, a non-SlHB8 binding site was found in the SlSPL promoter, indicating indirect regulation by SlHB8, a regulatory loop with miR166, or a loss of gene expression. DYT1, which is positively regulated by SPL and EMS1, functions downstream of SPL and partially rescues the spl phenotype [51]. DYT1 is sufficient to activate the downstream
The genetic pathway (DYT1–TDF1–AMS–MS188–MS1) for tapetum development is reported to be closely connected to exine formation in Arabidopsis. The key genes involved in sporopollenin formation, such as CYP703A2, CYP704B1, PKSB, and PKSA, are positively regulated by DYT1, TDF1, AMS, and MYB80 [23, 52]. Moreover, AMS regulates nixine and sexine layer formation by directly modifying the expression of TEK and MS188. The absence of TEK function results in pollen grains without the intine and exine layers [22]. In the present study, the exine and intine were affected in the p35s::SlHB8Ris lines, with an extine thicker than that of the WT and an absent intine (Fig. 4). 

Compared with exine development, knowledge of intine formation is lacking. Intine is secreted by the microspores and is associated with pectin, cellulose, and callose metabolism [21]. Inhibition of intine synthesis during the early stages of male gametogenesis may arrest pollen development, leading to collapsed, abortive pollen [53–55]. As the pollen tube consists of an intine layer, defects of the intine structure are accompanied by abnormal pollen tube germination [54, 56–58]. In the p35s::SlHB8Ris lines, an intine layer did not form in the shrunken, irregular, and infertile pollen grains and the germination rate was reduced (Fig. 4; Fig. 3a, Fig. S3d, see online supplementary material). UDP-sugar pyrophylase (USP) is involved in pectin synthesis; loss-of-function of AtUSP blocks the synthesis of matrix polysaccharides, which are required for intine synthesis, resulting in pollen without an intine layer [57]. The homologous gene of USP in tomato was found to be a direct target of SlHB8 (Table S5, see online supplementary material), indicating a complex regulation of this gene. FME genes encode enzyme called pectin methyltransferases which function in the de-esterification of pectin. FME genes belong to a multigene family, and some of them display a pollen-specific expression pattern. BcPME23c and BcMFT23a mutations cause abnormal thickening of the pollen intine of Bactris campestris, which affects pollen germination and growth [54, 56].
Figure 8. Validation of SIHB8 downstream target genes via yeast one-hybrid, EMSA and dual-luciferase assays. 

(a) Validation of SIHB8 binding with the four selected DAP-seq fragments using yeast one-hybrid assay. AD-empty indicates the control yeast strain transformed with the empty pGADT7 vector without SIHB8.

(b) EMSA validation of the binding motifs in four selected DAP-seq fragments. The corresponding motif are listed beside the band. The detected bands are indicated with black arrows.

(c) Validation of SIHB8 activation on five selected promoters using a dual-luciferase assay. The empty effector was used as control (set as 1). The ratio is presented as the mean ± SE (n = 3).

Figure 9. Schematic model of SIHB8-regulated genes in tapetum development and degradation.

To late pollen germination and lower germination rate [59]. In our study, four out of eight PME genes showed increased expression levels in the p35s::SIHB8Ris line (Fig. 7d), among which SPM8 was the target gene of SIHB8 (Table S5). Polygalacturonase (PG) – whose gene family is expressed in the pollen and/or anthers – functions in the pectin degradation and cell wall disintegration. BcMF2, BcMF6, and BcMF9 are associated with intine development. Inhibition of BcMF2 or BcMF9 results in reduced PG activity and disturbed cell wall metabolism in the process of intine formation [53]. In our study, two out of three PG genes showed increased expression levels in the p35s::SIHB8Ris line (Fig. 7d), among which SIS1 was the target gene of SIHB8 (Table S5, see online supplementary material). PLs encode Pectate lyases (or pectate transesterases; PLs) function in the process of cell wall disintegration and are necessary for intine loosening. Down-regulation of BcPLL19 led to abnormal intine formation and delayed pollen tube growth in B. campestris ssp. chinensis [60]. The reduced expression level of BcPLL20 resulted in abnormal disportionated of intine distribution [61]. In our study, two genes encoding the PLs (LAT56 and AT59) were down-regulated in the p35s::SIHB8Ris line (Fig. 7d), suggesting the potential regulation by SIHB8. Finally, the fasciclin-like arabinogalactan protein affects microspore development and intine formation through cellulose deposition. Down-regulation of FLA3 in plants reduces male fertility and produces collapsed pollen grains without an intine [55]. Antisense RNA transgenic lines with reduced BcMF18 levels show abnormal pollen grains lacking an intine, cytoplasm, and nuclei as well as abnormal cellulose distribution [62]. In our study, two FLA genes showed increased expression levels in the p35s::SIHB8Ris line (Fig. 7d), but none contained a SIHB8 binding site (Table S5, see online supplementary material), indicating indirect regulation. Until now, most studies on intine formation have focused on the enzymes involved in pectin, cellulose, and callose metabolism. SIHB8 is thus the first transcription factor demonstrated to exhibit direct regulation of the genes involved in intine formation.

In summary, SIHB8 exhibited space–time characteristic expression pattern from microsporocyte differentiation to the microspore generation (Fig. 1), phenotypes of the SIHB8 knockout and overexpression lines along with the RNA-seq and DAP-seq data support the notion that SIHB8 together with the conserved genetic pathway SPL–DYT1–TDF1–AMS–MS80 are instrumental in the regulation of tapetum development and degradation.
Loss-of-function of \textit{SlHB8} induces pollen activity and promotes fruit setting (Figs 2b and 3b,d), but the pollen morphology and tapetum degradation were similar to that of wild-type plant (Figs 3a, 4 and 5). Moreover, the expression levels of tapetum PCD regulators were not affected in the \textit{SlHB8cr} plant (Fig. S9c, see online supplementary material) indicating another partner may compensate the function of \textit{SlHB8} or work together with \textit{SlHB8} in regulating the PCD process. By contrast, \textit{SlHB8} overexpression induces the expression of these conserved pathway genes, leading to premature tapetum degradation and resulting in pollen abortion. Based on these findings, a putative regulatory mechanism was proposed (Fig. 9), where overexpression of \textit{SlHB8} resistant to miR166-induced SPL expression directly or indirectly activates DVT1, TDF1, and MYB80 expression, thereby accelerating tapetum development and degradation. Therefore, \textit{SlHB8} emerges as a factor regulating pollen development. These findings expand our understanding of the molecular factors involved in tapetum development and degradation and provide potential genes for breeding strategies aimed at controlling this important trait.

**Materials and methods**

**Plant material and growth conditions**

\textit{SlHB8} knockout mutants were generated using CRISPR/Cas9. One single guide (sg) RNA (GCAGAAGCAAGTTTCACAGT) complementary to the coding sequence (CDS) of Solyc08g066500 was constructed into the pAGM4723 vector and transformed into \textit{Agrobacterium tumefasciens}, which was used for tomato genetic transformation. Plants bearing two kinds of 8 bp deletions in the CDS and a 1 bp insertion in the CDS were obtained. The two 8 bp deletion lines were used for flower and fruit development studies. The overexpression lines p35s::\textit{SlHB8R} and p2A11::\textit{SlHB8R} were generated separately. The full-length CDS of \textit{SlHB8R} was cloned into the overexpression vectors pMDC32 and 2A11, which were under control of the 35 s and 2A1 promoters, respectively, that show specific expression during the mature stages of anther and early fruit development [63]. The transgenic plants were selected on MS medium with antibiotic selection of the construction vector. Positive overexpression lines were identified by checking the expression levels of \textit{SlHB8}.

**RNA in situ hybridization**

The anthers at MI, Tds, MUM, BM, MP stages of wild-type were sampled for RNA in situ hybridization analysis. The experiment was carried out with reference to the method described [46]. All images were taken using an optical microscope (Zeiss, Oberkochen, Germany).

**Quantitative reverse transcription (qRT)-PCR**

Total RNA was extracted, after which the PrimeScript TM RT reagent kit (Takara Bio, Kusatsu, Japan) was used for the cDNA synthesis. qRT-PCR was carried out with SYBR PrimeScript™ RT PCR Kit II (Takara Bio) and sequence-specific primers, with ubiquitin (UBI; serial number: Solyc01g056940) as the reference gene. The relative expression levels of examined genes were computed according to the $2^{-\Delta\Delta CT}$ method with three biological replicates.

**Fruit phenotype analysis**

Five plants were randomly selected, and then ten flowers from each plant were randomly selected for fruit set rate counting. The length and width of Br + 7 fruits (7 days after the breaker stage) were measured using a cursor caliper, and single fruit weight was determined using an electronic analytical balance. The number of seeds in the tomato fruits at the Br + 7 stage was also counted.

**Pollen viability assay**

Pollen viability was measured using the TTC method [26], and pollen germination media used was according to Yang’s method [26]. Tomato pollen from the wild-type (WT) and \textit{SlHB8} transgenic tomato plants were incubated in PGM at 25°C for 2 h. Images were taken under a Leica microscope (Leica, Wetzlar, Germany). Anthers from the WT and transgenic tomato plants were counterstained with 0.1 mg mL$^{-1}$ DAPI to assess their nuclear status. The DAPI emission signals were 350 nm/460 nm. WT and transgenic lines were crossed as both paternal and maternal plants, after which fruit setting rates were statistically analysed.

**Cytological characterization of anthers**

Anthers from different developmental stages (MI, microspore mother cell stage; Tds, tetrad stage; MUM, middle uninucleate microspore stage; BM, binucleate microspore stage; MP, mature pollen stage) were collected during the flowering period and fixed at room temperature for 24–36 h. After paraffin embedding and sectioning, anther cell characteristics were examined using a Leica microscope. A terminal deoxynucleotidyl transferase-mediated-biotin-16-dUTP nick-end labeling (TUNEL) assay was carried out using the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA), according to its handbook. The images of sections were taken under a Leica TCS SP5 fluorescence confocal scanning microscope. Emission wavelengths of 488 nm/505–545 nm and 561 nm/575–650 nm were used for the TUNEL and propidium iodide signals detection.

**Electron microscopy of pollen phenotypes**

For scanning electron microscopy (SEM), mature pollens from \textit{SlHB8} transgenic and wild-type plants were fixed on SEM carriers, coated with gold–palladium. Pollen images were taken under a EVO MA15 scanning electron microscope (Zeiss, Oberkochen, Germany).

Anthers at different developmental stages (MI, Tds, MUM, BM, and MP) were fixed in 4% glutaraldehyde and 2% paraformaldehyde at 4°C overnight. After washing with 0.1 M PBS (four times), the fixed pollen grains were incubated in 1.5% low melting agar, post-fixed in 1% osmium tetroxide, and dehydrated with a graded ethanol series. The samples were then transferred to acetone and embedded in Spurr’s resin (SPI, West Chester, PA, USA). Sections (70 mm thick) were cut using an ultramicrotome (UC7, Leica), collected on copper grids, and stained with uranyl acetate and lead citrate. The stained grids were then photographed with a Talos L120C electron microscope (Thermo Fisher Scientific, Waltham, MA, USA).

**RNA-seq and DNA affinity purification (DAP)-seq analysis**

Flower Buds at the tetrad stage were collected and frozen with liquid nitrogen for RNA extraction and transcriptome sequencing experiment which will be carried out by the company Gene Denovo Biototechnology Co., Ltd (Guangzhou, China). Fragments per kilobase of transcript per million mapped reads (FPKM) was used.
for calculating the expression levels of detected genes. The threshold of log2 (fold change) ≥ 1 and false discovery rate (FDR) ≤ 0.05 were used for defining the differentially expressed genes (DEGs). Transcriptome data analysis and mapping were performed using the online platform OmicShare Tools developed by Gene Denovo (www.omicshare.com/tools). Heatmaps were generated using TBtools as described in the manual [64].

The SlHB8 gene was cloned into the protein expression halo vector provided by Gene Denovo, the buds at different developmental stages (MI, Tds, MUM, BM, and MP) were graded sampled and mixed at equal ratios, frozen with liquid nitrogen for stock. Protein purification for DAP-seq and extraction and sequencing of genomic DNA were performed by Gene Denovo.

Yeast one-hybrid assay (Y1H)
The pGADT7-Rec vector with full length CDS of SlHB8 was treated as a prey vector. pAbAi vector containing multiple SlHB8 binding elements obtained from DAP-seq analysis were regarded as a bait vector. The selection of minimal inhibitory concentration of aureobasidin A was carried out after the transformation of Y1H Gold yeast strains with linearized pAbAi constructs. The binding activity of SlHB8 on the elements was examined by transforming the prey vector to the bait yeast strains which will be cultured on the SD medium lacking Leu (SD/−Leu) with or without aureobasidin A of selected concentration at 30°C for 2–3 days.

Dual-luciferase transient expression assay
To check the regulatory activity of SlHB8 on the promoters containing SlHB8 binding elements, the pGreenII 62-SK vector containing the SlHB8 CDS were treated as effector vector, and the pGreenII 0800-LUC vector with target promoters were regarded as reporter vector. These plasmids with differential ratios and composition were injected into Nicotiana benthamiana leaves via Agrobacterium tumefaciens mediation. The activities of luciferase and Renilla were measured using the Dual-Luciferase Assay Kit (Promega) according to its handbook.

Metabolite analysis
Anthers from flowers at the anthesis stage were collected, frozen in liquid nitrogen for stock. Primary metabolome analysis was performed by MetWare Biotech Ltd. ANOVA (P < 0.01) was used to identify different metabolomes between WT and SlHB8 transgenic plant.

Electrophoretic mobility shift assay (EMSA)
The pGEX-4 T-1 vector containing full-length CDS of SlHB8 was transferred into Escherichia coli strain BM Rosetta (DE3) to producing SlHB8-GST fusion protein. The SlHB8 protein purification and EMSA operational approach were according to the methods described in the publication of Drakakaki [65].

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Author contributions
C.W., Y.Y., Z.X., C.Y., Z.P., H.G., and D.S. performed the research. Y.H., R.C., and Z.L. design of the research. Y.H., G.H., D.C., and C.W. analysed the data. Y.H. and C.W. wrote the manuscript. All authors assisted with manuscript revision. All authors read and approved the final version of the manuscript.

Data availability
All data generated or analysed during this study are included in this published article and its supplementary information files.

Conflict of interest
The authors declare no conflicts of interest.

Supplementary data
Supplementary data is available at Horticulture Research online.

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