The \textit{GAMYB-like} gene \textit{SlMYB33} mediates flowering and pollen development in tomato

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Abstract

\textit{GAMYB} are positive GA signaling factors that exhibit essential functions in reproductive development, particularly in anther and pollen development. However, there is no direct evidence of the regulation of any \textit{GAMYB} in these biological processes in tomato (\textit{Solanum lycopersicum}). Here, we identified a tomato \textit{GAMYB-like} gene, \textit{SlMYB33}, and characterized its specific roles. \textit{SlMYB33} is predominately expressed in the stamens and pistils. During flower development, high mRNA abundance of \textit{SlMYB33} is detected in both male and female organs, such as microspore mother cells, anthers, pollen grains, and ovules. Silencing of \textit{SlMYB33} leads to delayed flowering, aberrant pollen viability, and poor fertility in tomato. Histological analyses indicate that \textit{SlMYB33} exerts its function in pollen development in the mature stage. Further transcriptomic analyses imply that the knockdown of \textit{SlMYB33} significantly inhibits the expression of genes related to flowering in shoot apices, and alters the transcription of genes controlling sugar metabolism in anthers. Taken together, our study suggests that \textit{SlMYB33} regulates tomato flowering and pollen maturity, probably by modulating the expression of genes responsible for flowering and sugar metabolism, respectively.

Introduction

\textit{GAMYB} encodes an R2R3-MYB transcription factor that acts as a positive gibberellin (GA) signaling factor\textsuperscript{1}. The first \textit{GAMYB} was isolated from aleurone cells in barley (\textit{Hordeum vulgare}) and controls the expression of most GA-inducible genes\textsuperscript{2-4}. \textit{GAMYB} has been shown to be a target of microRNA 159 (miR159), a highly conserved miRNA family in the common ancestor of all embryophytes\textsuperscript{5}. MiR159 controls the cleavage of \textit{GAMYB} mRNA\textsuperscript{6-8}.

A series of studies have reported the differing roles of \textit{GAMYBs} in the regulation of flowering. \textit{Lolium temulentum LIGAMYB} is mainly expressed in the shoot tip, and its transcript level is upregulated in parallel with an increased GA content during the floral transition\textsuperscript{9}. The \textit{GAMYB} family has three members in \textit{Arabidopsis}: \textit{AtMYB33}, \textit{AtMYB65}, and \textit{AtMYB101}\textsuperscript{10}. When \textit{Arabidopsis} plants are transferred from short-day to long-day conditions or treated with exogenous GA under short days, flowering occurs, and the expression of \textit{AtMYB33} and \textit{LEAFY} (\textit{LFY}), the floral meristem-identity gene, increases in the shoot apex at that time. Furthermore, the expression timing and pattern of \textit{AtMYB33} precede and overlap with those of \textit{LFY} at the shoot apex, and \textit{AtMYB33} can bind to a specific 8-bp sequence in the \textit{LFY} promoter\textsuperscript{10}. These observations imply that \textit{GAMYB} may be involved in GA-regulated flowering via the transcriptional activation of the \textit{LFY} gene. However, several studies have suggested that loss-of-function mutants of \textit{GAMYBs} do not exhibit altered flowering in \textit{Arabidopsis} and rice (\textit{Oryza sativa})\textsuperscript{11,12}. Moreover, the manipulation of miR159 controls flowering time in some cases by regulating the expression level of its target \textit{GAMYB}. For instance, the overexpression of miR159 delays flowering by inhibiting \textit{AtMYB33} under a short-day photoperiod in \textit{Arabidopsis}\textsuperscript{8}, but another line of evidence showed that the...
flowering time remains normal under long-day conditions in miR159-overexpressing plants, which causes the downregulation of \( \text{AtMYB101} \). In contrast, the \( \text{Arabidopsis} \) \( \text{mir159ab} \) double mutant displays late flowering under short days, but shows no alteration of flowering time under long days. In addition, the repression of miR159, which increases \( \text{GAMYB} \) expression, accelerates flowering in gloxinia (\( \text{Simmingia speciosa} \)) but delays flowering in tobacco (\( \text{Nicotiana tabacum} \)).

\( \text{GAMYB} \)s also play an essential role in flower development, particularly in stamen development. In barley, the expression of \( \text{HvGAMYB} \) is predominately detected in the anthers. However, single mutants result in anther failure, anther dehiscence, and male sterility, mimicking the effect of excessive GA on flower development. In rice, \( \text{OsGAMYB} \) is strongly expressed in the shoot apex, stamen primordia, and tapetum cells at the reproductive stage. Loss of function of \( \text{OsGAMYB} \) results in some defects in anther and pollen development. Moreover, \( \text{OsGAMYB} \) has been found to participate in the GA-regulated formation of exines and Ubisch bodies and the programmed cell death (PCD) of tapetal cells; specifically, the direct induction of \( \text{CYP703A3} \) by \( \text{OsGAMYB} \) is key to the formation of exines and Ubisch bodies. The \( \text{Arabidopsis} \) \( \text{myb33 myb65} \) double mutant shows shorter stamens that fail to fully extend to the stigma, and displays defective pollen development owing to the hypertrophy of the tapetum at the microspore mother cell (MMC) stage, resulting in male sterility. However, single mutants (\( \text{myb33} \) or \( \text{myb65} \)) do not exhibit aberrant phenotypes, indicating crucial functional redundancy between \( \text{AtMYB33} \) and \( \text{AtMYB65} \). The overexpression of miR159, repressing its target \( \text{AtMYB33} \), also causes anther defects and male sterility.

There are three \( \text{GAMYB} \)-like genes in cucumber (\( \text{Cucumis sativus} \)): \( \text{CsGAMYB1} \), \( \text{CsGAMYB2} \), and \( \text{CsGAMYB3} \). The silencing of \( \text{CsGAMYB1} \) decreases the number of male flower nodes and increases that of female flower nodes, leading to a significant change in cucumber sex expression. In tomato, the \( \text{GAMYB} \) homolog \( \text{SlGAMYB1} \) has been identified, which is expressed in the embryo and endosperm during seed germination and in young vegetative tissues.

Therefore, although \( \text{GAMYB} \)s have been studied extensively in plants, there is no direct evidence of the regulation of any \( \text{GAMYB} \) (including \( \text{SlGAMYB1} \)) during flowering and pollen development in tomato, which is an important commercial crop and a model species for studying flower and fruit development. Therefore, in the present study, we revealed the specific roles of \( \text{SIMYB33} \), a \( \text{GAMYB} \)-like gene, in flowering and pollen development in tomato. We found that \( \text{SIMYB33} \) was mainly expressed in stamens and pistils. The knockdown of \( \text{SIMYB33} \) resulted in delayed flowering, aberrant pollen maturity, and remarkably decreased fertility. RNA-Seq analyses revealed that \( \text{SIMYB33} \) probably achieves its positive roles in tomato flowering and pollen maturity by regulating the expression of genes involved in flowering and sugar metabolism, respectively.

**Results**

Identification of the \( \text{GAMYB} \)-like gene \( \text{SIMYB33} \) from tomato

Following a BLAST search in the Solanaceae Genomics Network, we identified two \( \text{GAMYB} \)-like genes, \( \text{SlGAMYB1} \) (\( \text{Solyc01g009070} \)) and \( \text{SIMYB33} \) (\( \text{Solyc06g073640} \)), in tomato. Since the \( \text{SlGAMYB1} \) gene has been reported previously, we focused on \( \text{SIMYB33} \) in this study. \( \text{SIMYB33} \) consists of three exons and two introns (Fig. 1a), similar to the \( \text{GAMYB} \) orthologs found in barley, \( \text{Arabidopsis} \), and cucumber. The full-length CDS of \( \text{SIMYB33} \) consists of 1515 bp and encodes 504 amino acids. Previous studies have reported that \( \text{GAMYB} \)s belong to the R2R3-MYB family and contain helix-turn-helix repeats R2 and R3 and three typically conserved motifs, Box 1, Box 2, and Box 3. Analyses of the protein domain structure and the sequence alignments of the amino acid residues revealed that \( \text{SIMYB33} \) also includes an R2R3-repeat DNA-binding domain and conserved Box 1 (\( \text{QRAGLPIYPSD} \)) and Box 2 (\( \text{NSGLLDAVLHESQ} \)) motifs, but no Box 3 (Fig. 1a; Supplementary Fig. S1). \( \text{SIMYB33} \) shares the highest overall identity with cucumber \( \text{CsGAMYB1} \) (40.49%); however, within the R2R3 sequence alone, the identity of \( \text{AtMYB33} \), \( \text{AtMYB65} \), \( \text{CsGAMYB1} \), and \( \text{HvGAMYB} \) with \( \text{SIMYB33} \) is 84.62%, 83.65%, 90.38%, and 89.42%, respectively (Supplementary Fig. S1).

To reveal the evolutionary relationships between \( \text{SIMYB33} \) and other \( \text{GAMYB} \) homologs, 32 predicted \( \text{GAMYB} \) proteins from 22 species were included in a phylogenetic analysis. As shown in Fig. 1b, the \( \text{GAMYB} \)s of Solanaceae species, including tomato, potato (\( \text{Solanum tuberosum} \)), pepper (\( \text{Capsicum annuum} \)), \( \text{Petunia hybrida} \), and \( \text{Caffea canephora} \), were placed in a single group, suggesting that they may share a common origin. Among these sequences, \( \text{SIMYB33} \), \( \text{Solanum pennellii} \) (a wild tomato species) \( \text{GAMYB} \)-like, and potato \( \text{GAMYB2} \) fall into the same clade, which is highly distinct from that of \( \text{Arabidopsis MYB33} \) and \( \text{MYB65} \). In addition, \( \text{SlGAMYB1} \) is located in the same cluster with \( \text{Solanum pennellii} \) \( \text{GAMYB} \) and potato \( \text{GAMYB1} \). These results implied that tomato \( \text{SIMYB33} \) belongs to the \( \text{GAMYB} \) family.

**\( \text{SIMYB33} \) expression pattern in tomato**

To address the possible functions of \( \text{SIMYB33} \), we first evaluated its expression profiles in various tissues of tomato (\( \text{Micro-Tom} \)) through qRT-PCR. \( \text{SIMYB33} \) is
mainly expressed in stamens and pistils, in which its transcript levels are at least fourfold higher than those in the seeds, roots, stems, leaves, sepals, petals, and fruits (Fig. 2a). This result was validated in the TomExpress database, and we found that the expression pattern of SlMYB33 showed a similar trend to the results of the qRT-PCR analysis (Supplementary Fig. S2). We also obtained expression data for another tomato GAMYB gene, SIGAMYB1, from TomExpress. SIGAMYB1 is highly expressed in flower buds compared with other tissues (Supplementary Fig. S2). These observations pointed to a potential role of SlMYB33 in tomato flower
development, and **SIGAMYB1** may have a similar function.

Next, we performed in situ hybridization to analyze the spatial/temporal expression of **SlMYB33** during tomato flower development in detail (Fig. 2b–l). **SlMYB33** transcripts were detected in the developing sepals, petals, stamens, carpels, and placenta primordia in early stages (stages 4 and 6, Fig. 2b, c)\(^{24}\), after which **SlMYB33** expression was restricted to the stamens and placentae (stage 7, Fig. 2d). In later developmental stages, **SlMYB33** was persistently expressed in the developing microspore mother cells (MMC), anthers, pollen grains, and ovules (stages 9–14, Fig. 2e–h). As negative controls, no signals were detected under **SlMYB33** sense probe hybridization (Fig. 2i–l). Our results suggested that **SlMYB33** may play an important role in both male and female gametophyte development in tomato.

**Knockdown of SlMYB33 results in delayed flowering in tomato**

To determine the biological function of **SlMYB33**, we attempted to inhibit its expression in Micro-Tom plants.
using gene-silencing technology. An RNA interference (RNAi) construct under the control of the CaMV 35S promoter was generated and transformed into the tomato plants. Following PCR analysis, ten independent SlMYB33-RNAi lines were obtained. Among these lines, seven displayed significant reductions in SlMYB33 transcript abundance by 60–92% in the flowers, whereas no apparent changes in SlGAMYB1 expression were observed (Fig. 3a), suggesting that only SlMYB33 was effectively knocked down. Due to the lower SlMYB33 expression levels in lines 3, 5, and 10 compared with the other lines, these three lines were chosen for further analysis (Fig. 3b).

Previous studies have reported the potential effect of GAMYB genes on flowering\textsuperscript{6,9,10}, therefore, the flowering time in the T\textsubscript{1} generation of SlMYB33-RNAi lines was recorded. For each RNAi line, 18 T\textsubscript{1} plants exhibiting apparent suppression of SlMYB33 were selected for this analysis (Supplementary Fig. S3). We found that flowering was initiated in the progenies of three transgenic lines after the emergence of 7.2–9 leaves, compared with 5.3 leaves in WT (wild-type) plants (Fig. 3c). Accordingly, the first flower opened at 50–56 days after sowing in the progenies of SlMYB33-RNAi lines, which was later than the time of 40.3 days observed in the WT plants (Fig. 3b, d). Moreover, the extension of the flowering time in the RNAi lines was negatively correlated with SlMYB33 expression; for instance, the lowest SlMYB33 mRNA level in the RNAi-10 line led to the most severe late-flowering phenotype (Fig. 3c; Supplementary Fig. S3). In addition, we investigated the flowering time of T\textsubscript{1} plants of three null SlMYB33-RNAi lines (RNAi-4, RNAi-7, and RNAi-9).

![Fig. 3 SIMYB33-RNAi results in delayed flowering in tomato.](image-url)
that displayed no obvious repression of SlMYB33 (Supplementary Fig. S4a). No significant changes in the numbers of leaves produced before the first flower were found in these transgenic lines compared with WT plants (Supplementary Fig. S4b). These data suggested that SlMYB33 can promote flowering in tomato.

Suppression of SlMYB33 leads to aberrant pollen maturity and poor fertility

To evaluate whether SlMYB33 could affect anther and pollen development similarly to a typical GAMYB homolog7,12,16,17, the pollen phenotypes were analyzed in the SlMYB33-RNAi lines. As expected, a majority of the pollen grains were aberrant in the RNAi lines (Fig. 4). Here, the in vitro pollen germination assay indicated that 65–88% of the pollen grains in the SlMYB33-RNAi lines had lost germination ability, compared with only 17% in the WT (Fig. 4a, d, g). Further microscopic observations by SEM revealed that the pollen grains from the RNAi lines were shrunken and collapsed compared with those of the WT (Fig. 4b, c, e, f). Quantitative analyses showed that 70–86% of the transgenic pollen grains displayed an abnormal morphology, whereas this percentage was only 3% in the WT (Fig. 4h).

To dissect how SlMYB33 regulates pollen development, histological analyses of flower buds at different stages were performed in WT and RNAi plants (Fig. 5). Among the five stages of tomato pollen development24, no evident abnormalities were found in the microspore mother cells (MMCs), tetrads, or uninucleate microspores in the anthers of RNAi-10 plants (Fig. 5a–c) compared to those of the WT (Fig. 5f–h). During the binucleate stage, the transgenic pollen grains were slightly shrunken and irregular but showed no defects in their structures (Fig. 5d, i). However, a striking phenotype was detected at the mature stage. In contrast to the WT, most of the mature pollen grains in RNAi-10 were severely collapsed and shrunken, which may be due to the degradation of the cytoplasm (Fig. 5e, j). In addition, there was no obvious change in transgenic tapetum development (Fig. 5), which can be mediated by GAMYB homologs in other species such as Arabidopsis and rice7,11,17.

Along with aberrant pollen development (Figs. 4 and 5), the suppression of SlMYB33 leads to a significant reduction in the fertility of tomato. As shown in Fig. 6, each fruit of the SlMYB33-RNAi lines produced only 3.1–7.3 seeds, which was a markedly lower number than 22.9 seeds recorded in the WT (Fig. 6b, c). Fruit size was also obviously decreased in the transgenic plants (fruit width of 13.7–17.5 mm, length of 13.0–16.7 mm) compared with the WT (fruit width of 22.7 mm, length of 20.8 mm), and was quantitatively related to seed number (Fig. 6a, d). Therefore, we speculated that this change in fruit size might be caused by the reduced seed number. In conclusion, the repression of SlMYB33 disrupts pollen maturity, resulting in poor fertility accompanied by smaller fruit in tomato.
Fig. 5 Suppression of *SIMYB33* disturbs pollen maturity. Transverse sections of flower buds at the microspore mother cell stage (a, f), tetrad stage (b, g), uninucleate microspore stage (c, h), binucleate microspore stage (d, i), and mature pollen stage (e, j) in WT and RNAi-10. MMC microspore mother cell, T tapetum, Tds tetrads, UMsp uninucleate microspore, BP binucleate pollen, MP mature pollen. Bars = 50 μm.

Fig. 6 The suppression of *SIMYB33* affects the fertility and fruit size of tomato. a Fruits of WT and *SIMYB33*-RNAi lines. b Transverse sections of the fruits of WT and *SIMYB33*-RNAi lines. c Numbers of seeds per fruit in WT and different RNAi lines. d Fruit sizes in WT and different RNAi lines. Values are the means ± SD of three independent plants, and 15 fruits were examined for each plant. Asterisks indicate significant differences between the RNAi lines and the WT by Student’s t test (**P < 0.01). Bars = 1 cm.
Knockdown of SIMYB33 restricts the expression of genes controlling flowering

To identify the potential genes and molecular pathways involved in SIMYB33-regulated tomato flowering, transcriptome analysis was performed in shoot apices (30-day-old, not flowering) from the SIMYB33-knockdown line RNAi-10 and WT plants by the digital gene expression (DGE) approach. Using a false discovery rate (FDR) < 0.05 and a fold change (FC) > 2 as significance cutoffs, we identified 2388 differentially expressed genes (DEGs), among which 1689 genes were upregulated, and 699 genes were downregulated in the RNAi-10 shoot apices compared with those of the WT (Supplementary Table S1, Supplementary Table S2). Through careful examination, we observed that the expression levels of several genes responsible for tomato flowering were dramatically decreased in the shoot apices of the RNAi lines, including the ANANTHA (AN), FALSIFLORA (FA), COMPOUND INFLORESCENCE (S), and SPGB genes, encoding the F-box protein UNUSUAL FLORAL ORGANS (UFO), the transcription factor LFY, the homeobox transcription factor WUSCHEL-HOMEBOX9 (WOX9), and the basic region/leucine zipper (bZIP) transcription factor SP-INTERACTING G-BOX (SPGB), respectively (Fig. 7a; Supplementary Table S2). The tomato homologs of two genes promoting flowering in Arabidopsis and rice, FLOWER-PROMOTING FACTOR1 (FPF1) and FLOWERING TIME CONTROL LOCUS A (FCA), were also significantly downregulated (Fig. 7a; Supplementary Table S2). qRT-PCR analysis was performed for these six DEGs, and the results revealed the same expression pattern as the RNA-Seq analysis. Therefore, the AN, FA, S, SPGB, FPF1, and FCA genes may be involved in SIMYB33-regulated flowering in tomato. Given the ability of GAMYM to bind to the promoters of target genes, we further analyzed the cis-acting elements in the promoters of these six DEGs. As shown in Fig. 7b, the promoters of the FA and S genes contained four and two MYB-binding sites, respectively, which were highly conserved with the 8-bp GAMYM-binding site (CAACGTTC) with a C/T/AAC core in Arabidopsis, implying that FA and S are putative candidate target genes of SIMYB33 in the regulation of tomato flowering.

SIMYB33 affects the expression of sugar metabolism genes

We also carried out RNA-Seq analysis to explore the possible gene networks through which SIMYB33 regulates tomato pollen maturity in anthers from the transgenic RNAi-10 and WT plants. Given that gene expression differences usually occur before phenotypic changes, the anthers were harvested before the mature pollen stage (late binucleate microspore stage) and used for transcriptome analysis. Through the DGE approach, a total of 1332 DEGs, including 676 upregulated genes and 656 downregulated genes, were identified (Supplementary Tables S1 and S3). Therefore, KEGG (Kyoto Encyclopedia of Genes and Genomes) classification was performed. The DEGs were assigned to 20 functional categories, among which “carbohydrate metabolism” was the most represented pathway, including 41 DEGs (Fig. 8a). Carbohydrate metabolism was then classified into 13 subgroups (Fig. 8b), among which starch and sucrose metabolism are essential for pollen development, especially for pollen maturity. Further examination revealed a dramatic reduction in the transcript levels of most of the genes grouped into the starch and sucrose metabolism pathways (Table 1). For instance, Lin7, encoding cell wall invertase (CW1N), which hydrolyzes sucrose into glucose and fructose, was significantly downregulated by 21.5-fold in the transgenic anthers compared with the WT. In addition, the expression levels of the following genes were obviously decreased: one gene encoding sucrose-phosphate synthase (SPS), which is a key regulator of sucrose synthesis, two genes that encode sucrose synthase (SUS), responsible for sucrose degradation, and one gene encoding trehalose-6-phosphate synthase (TPS), involved in the production of trehalose-6-phosphate (T6P), which acts as a signaling molecule in sensing sucrose availability and promoting starch and cell wall biosynthesis. We further found that the transcripts of two genes encoding 6-phosphofructokinase and fructose-bisphosphate aldolase, which are two key enzymes in the glycolysis pathway, were depressed in the RNAi line. Moreover, a gene for β-glucosidase (GLU), which can release glucose from the inactive gluco-side, is upregulated. In addition, the gene for ADP-glucose pyrophosphorylase (AGPase), a key enzyme for starch synthesis, exhibited a reduced mRNA level; however, a similar expression profile was detected in the β-amylase gene responsible for starch degradation. In addition, the expression of the gene (no classification) encoding sugar transporter protein 13 (SISTP13) was remarkably inhibited (Table 1). qRT-PCR assays of the above genes showed the same expression trend as the RNA-Seq analysis (Supplementary Fig. S5). These results indicated that the silencing of SIMYB33 disrupts tomato pollen maturity at least partly by repressing the transcription of genes related to starch and sucrose metabolism as well as sugar transport.

Furthermore, we observed remarkable expression differences in several genes (belonging to the “pentose and glucuronate interconversions” pathway) controlling cell wall degradation in transgenic RNAi-10 anthers compared with those of the WT. Here, two pentiesterase (PE) genes and one pectate lyase (PL) gene displayed upregulation, whereas one polygalacturonase (PG) gene showed downregulation (Table 1; Supplementary Fig. S5), revealing the possible involvement of these genes in pollen collapse in SIMYB33-RNAi plants.

We then performed metabolite analysis to examine whether the changes in the expression of genes related to
sugar metabolism could influence the contents of carbohydrates. As expected, we found that the knockdown of SlMYB33 significantly decreased the sucrose content by 6.2-fold in anthers at the mature pollen stage, and the concentrations of glucose and fructose also declined in the RNAi-10 plants (Fig. 9).

**Discussion**

**GAMYB functions in the regulation of flowering**

Our results suggested that the knockdown of SlMYB33 delays tomato flowering (Fig. 3). However, there are differing reports about the roles of GAMYBs in the regulation of flowering. Overexpression of miR159 in Arabidopsis ecotype Landsberg erecta causes late flowering through the downregulation of the target AtMYB33 under short days; however, in Columbia, the overexpression of miR159, repressing AtMYB101, does not alter flowering time under long days. It has also been reported that the loss of function of GAMYBs in Arabidopsis (Columbia background) and rice does not affect flowering; moreover, the transcriptomes of the shoot apices are almost identical between WT and myb33 myb65 mutant
in Arabidopsis\textsuperscript{11}. Nevertheless, in gloxinia, the overexpression or repression of miR159 causes the downregulation or upregulation of \textit{GAMYB}, leading to delayed or early flowering, respectively\textsuperscript{13}. In contrast, a recent report demonstrated that transgenic tobacco plants with greater \textit{GAMYB} levels exhibit a late-flowering

#### Table 1  Differentially expressed genes in the anthers of \textit{SIMYB33-RNAi} and WT plants

| Functional category | Gene ID       | Gene annotation                          | FC   | FDR       |
|---------------------|---------------|------------------------------------------|------|-----------|
| Starch and sucrose metabolism | Solyc09g010090 | Cell wall invertase (Invertase 7, Lin7)  | −21.53 | 2.76E−05 |
|                     | Solyc11g045110 | Sucrose-phosphate synthase (SPS)         | −10.13 | 2.78E−02 |
|                     | Solyc07g042550 | Sucrose synthase (SUS3)                  | −4.02  | 4.10E−02 |
|                     | Solyc12g040700 | Sucrose synthase                        | −3.19  | 4.23E−05 |
|                     | Solyc02g071590 | Trehalose-6-phosphate synthase (TPS)     | −2.07  | 7.14E−03 |
|                     | Solyc04g015200 | 6-Phosphofructokinase 2                  | −2.06  | 8.53E−06 |
|                     | Solyc07g065900 | Fructose-bisphosphate aldolase           | −3.88  | 2.43E−04 |
|                     | Solyc08g044510 | β-Glucosidase (GLU)                     | 3.28   | 3.28E−02 |
|                     | Solyc12g011120 | ADP-glucose pyrophosphorylase (AGPase)   | −3.51  | 1.49E−02 |
|                     | Solyc09g091030 | β-Amylase 1                             | −3.10  | 3.37E−02 |
|                     | Solyc03g005150 | Sugar transporter protein 13 (SISTP13)   | −4.15  | 4.47E−02 |
| Pentose and glucuronate interconversions | Solyc06g009190 | Pectinesterase (PE)                   | 2.47   | 6.27E−05 |
|                     | Solyc05g047590 | Pectinesterase (PE)                     | 2.02   | 3.56E−02 |
|                     | Solyc03g111690 | Pectate lyase (PL)                      | 2.08   | 2.56E−02 |
|                     | Solyc06g068040 | Polygalacturonase (PG)                   | −3.28  | 4.95E−03 |

Fig. 8 KEGG classification of DEGs in anthers between RNAi-10 and WT. a Functional categories of DEGs, b Secondary classification of the carbohydrate metabolism pathway
phenotype. These observations indicated that the functions of GAMYBs in flowering appear to be determined by a complex mechanism, which may be affected by differences in species or ecotypes. This is the first time that the direct downregulation of GAMYB has been shown to result in late flowering, providing novel evidence of the diversity of GAMYB functions in flowering.

**SIMYB33 shows conserved and different roles in pollen development compared with its homologs**

GAMYBs have been verified to positively regulate stamen development. Loss of function of GAMYBs leads to defects in stamen, anther, and pollen development, resulting in male sterility in Arabidopsis and rice. In our study, expression analyses showed that tomato SIMYB33 is highly expressed in staminal organs (Fig. 2). The knockdown of SIMYB33 results in aberrant and inviable pollen grains (Fig. 4) and ultimately poor fertility (Fig. 6). Although fruit size also decreases in SIMYB33-RNAi lines, this may be a consequence of the reduced seed number in fruits (Fig. 6). These data supported the notion that SIMYB33 exhibits a conserved function in the promotion of pollen development.

Moreover, Arabidopsis myb33 myb65 and rice gamyb mutants show abnormal pollen development, owing to the loss of programmed cell death (PCD) and consequent endless hypertrophy of the tapetum, demonstrating the importance of GAMYB in tapetum degradation. However, we did not find any obvious change in tapetum morphology at various developmental stages in SIMYB33-RNAi anthers compared with those of the WT, while the abortion of transgenic pollen grains was caused by damaged pollen maturity (Fig. 5), indicating that SIMYB33 plays a key role in tomato pollen maturity, rather than tapetum development. Therefore, despite the positive effect of both SIMYB33 and its homologs on pollen development, their regulatory mechanisms are different.

The potential effect of BR–GA crosstalk in phenotypes caused by the knockdown of SIMYB33

The tomato cultivar Micro-Tom was used as the studied plant material in this work. This genotype is a brassinosteroid (BR)-deficient mutant that exhibits a very dwarf phenotype with small fruits, but with no effect on flowering. Further study suggested that BR and GA synergistically mediate vegetative growth in Micro-Tom. BRs and GAs are two groups of growth-promoting phytohormones that can interact during many developmental processes. BR induces GA biosynthesis to regulate plant growth, affecting processes such as cell elongation in rice and seed germination, hypocotyl elongation, and flowering in Arabidopsis. Damagalska et al. also reported that the promotion effect of BR on flowering depends on the presence and concentration of GA in Arabidopsis. On the other hand, GA can regulate plant growth by modulating BR biosynthesis and signaling. SPINDLY (SPY), a negative regulator of GA signaling, inhibits BR biosynthesis to mediate lamina joint bending, whereas GERMOSTATIN RESISTANCE LOCUS 1 (GSR1), a positive regulator of GA signaling, stimulates BR biosynthesis to control root and leaf development and fertility in rice. Moreover, DELLA proteins, which are the key repressors of GA signaling, interact with the positive regulators of BR signaling BRASSINAZOLE-RESISTANT1 (BZR1)/BRASSINAZOLE-RESISTANT1 (BRI1)/EMS-SUPPRESSOR1 (BES1) and repress their activities. Therefore, GA can release the DELLA-modulated inhibition of BZR1/BES1 to induce BR responses and plant growth.

In this work, phenotypic differences were observed between SIMYB33-RNAi lines and WT plants of the same genetic background (Micro-Tom), indicating that the phenotypes were caused by the silencing of SIMYB33, rather than BR deficiency in Micro-Tom. However, given the general feedback regulation of GA production by GA signaling, together with the complexity of BR–GA interaction, it remains possible that the knockdown of SIMYB33 affects the crosstalk between BR and GA at the...
biosynthesis level and/or the signaling level, resulting in late flowering and abnormal pollen development.

**Flowering-related genes are candidate genes regulated by SIMYB33**

Our data revealed the important finding that the knockdown of SIMYB33 affects the expression of some genes controlling flowering, including FA, AN, S, SPGB, FPF1, and FCA (Fig. 7a). FA is an ortholog of Arabidopsis LFY, which is responsible for flower initiation26. Previous studies have demonstrated that the LFY can be activated by the application of GA, while GAMYBs influence flowering by regulating the GA responsiveness of the LFY promoter in Arabidopsis10,59. Likewise, FA can mediate tomato flowering, as demonstrated by a late-flowering phenotype with an increased number of leaves before the first inflorescence in the fa mutant26. AN encodes an F-box homolog of Arabidopsis UFO, while the S gene encodes a WOX9 transcription factor. S and AN are sequentially expressed during the phase transition from the inflorescence meristem to the floral meristem, and play overlapping roles in inflorescence architecture as well as floral identity. A mutation in either AN or S delays tomato flowering, leading to highly branched inflorescences21,27. Genetic interaction analysis shows that AN functions downstream of FA, demonstrating the crucial function of FA in flowering27. Here, our work revealed that the silencing of SIMYB33 results in late flowering (Fig. 3), and significantly reduces the transcript levels of FA, AN, and S (Fig. 7a). In addition, the promoters of the FA and S genes contain putative GAMYB-binding sites (Fig. 7b). Based on these findings, we speculated that SIMYB33 controls flowering partly via the transcriptional activation of the FA–AN pathway and the S gene. Between the FA and S candidates, the former is most likely a downstream target of SIMYB33, as indicated by the homology of SIMYB33 and FA to Arabidopsis AtMYB33 and LFY, respectively (Fig. 1; Supplementary Fig. S1)36, while AtMYB33 functions upstream of LFY60.

**SIMYB33 probably modulates pollen maturity by regulating the expression of sugar metabolism genes**

Our work also highlighted a potential role of SIMYB33 in mediating the expression of genes related to sugar metabolism (Fig. 8; Table 1), which is necessary for pollen development36,37. For pollen to be viable, the import of sufficient nutrients is essential to support a series of physiological activities. CWIN hydrolyzes sucrose into glucose and fructose, which are then taken up into pollen by hexose transporters following an apoplastic pathway39,40,66. Previous studies have reported the crucial role of CWIN in pollen development. The downregulation of tobacco CWIN results in inviable pollen because of the loss of starch and cell wall integrity61. In addition, pollen sterility is attributable to a reduction in the expression of CWIN together with hexose transporter genes under cold stress in rice62. Accordingly, we identified a dramatic decrease in the expression of the tomato CWIN ortholog Lin7 (SICWIN3)60, in SIMYB33-RNAi anthers (Table 1). Lin7 has been reported to be predominately expressed in tomato anthers65; however, its biological function is still elusive. Chen et al.38 speculated that Lin7 potentially plays a role in postmeiotic pollen development, while our data revealed a possible effect of Lin7 on pollen maturity in tomato, suggesting that the function of CWIN homologs in pollen development may be conserved. Moreover, one SPS and two SLIS genes were obviously downregulated in transgenic anthers (Table 1). SPS is a key enzyme for sucrose synthesis, whereas SUS contributes to sucrose cleavage to generate UDP-glucose and fructose40. Likewise, SISTP13, a sugar transporter gene, was inhibited in the transgenic lines (Table 1). STPs serve as hexose/H+ symporters and contribute to the transport of glucose and fructose from the cell wall to the cytoplasm63.

Starch is highly important as an energy source in the microsporangium, and is essential for pollen ontogeny38. AGPase catalyzes the first key step of starch biosynthesis65,66. Strikingly, SPS activity is positively correlated with starch accumulation67. Therefore, the downregulation of AGPase and SPS (Table 1) indicated that the starch concentration might be decreased in SIMYB33-RNAi anthers. Moreover, a reduced mRNA level of one gene encoding β-amylase was observed (Table 1); this enzyme is an exoenzyme responsible for starch cleavage to release maltose molecules38, suggesting that starch degradation is also influenced.

In conclusion, the abnormal pollen maturity observed in SIMYB33-knockdown plants might be caused at least in part by the restriction of sucrose and starch metabolism and sugar transport, leading to a lack of nutrient reserves in pollen grains. The decreased sucrose, glucose, and fructose contents detected in RNAi-10 anthers at the mature pollen stage (Fig. 9) provided further evidence to support this proposition.

**The possible role of miR159 in SIMYB33-regulated flowering and pollen development**

Through BLAST analysis with mature miR159 sequences from Arabidopsis in mirBase (http://www.mirbase.org)60,68, we found two miR159 members in tomato: miR159a (MIMAT0009141) and miR159b (MIMAT0042036) (Supplementary Fig. S6a). A binding site was then detected between SIMYB33 and miR159a/b (Supplementary Fig. S6b), suggesting that SIMYB33 may act as the target of miR159a and miR159b.

Previous reports have indicated that miR159 functions in a feedback-regulatory loop with GAMYB in Arabidopsis in which miR159 directs the downregulation of
GAMYB activity and is then compensatorily upregulated by GAMYB. Here, we performed comparative expression analyses of miR159a and miR159b between SIMYB33-knockdown and WT plants; however, no significant changes were observed in either the shoot apices or anthers (Supplementary Fig. S6c), implying that there may be no feedback regulation of miR159 by SIMYB33 in tomato.

Based on the above observations and the high conservation of the miR159 family, we predicted that SIMYB33-regulated flowering and pollen development may be controlled by miR159 (a/b) in tomato, but without the involvement of a feedback-regulatory mechanism.

Materials and methods

Plant materials and growth conditions

Tomato (Solanum lycopersicum L. cv Micro-Tom) seeds were germinated in a petri dish at 28 °C in the dark for 3 days, and the seedlings were then cultured in a growth room under a 16-h/8-h photoperiod with day/night temperatures of 25 °C/18 °C. Water management and pest control were performed according to standard practices.

SIMYB33 cloning, sequence alignment, and phylogenetic analysis

Total RNA was extracted from tomato flower buds using an RNA extraction kit (TaKaRa, Kusatsu, Japan), and cDNA was synthesized using the PrimeScript RT reagent kit (TaKaRa). The coding sequence (CDS) of SIMYB33 was amplified by PCR using gene-specific primers (Supplementary Table S4). The protein domain structure of SIMYB33 was analyzed using the software DOG 2.0.

The amino acid sequences of related GAMYB proteins in various species were obtained from the Solanaceae Genomics Network (http://www.solgenomics.net), National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), or Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html) database. Then, multiple-sequence alignment was carried out using MEGA5 software, and boxes highlighting conserved sequences were drawn using the online software BoxShade (http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic analysis was conducted via the neighbor-joining method with MEGA5, and bootstrapping analyses were performed with 1000 replications.

qRT-PCR analysis

For the gene expression assay, total RNA extraction and cDNA synthesis were performed using an RNA extraction kit (TaKaRa, Kusatsu, Japan) and a PrimeScript RT reagent kit (TaKaRa), respectively. Then, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was carried out using the SYBR Premix Ex Taq kit (TaKaRa) with an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, CA, United States). The tomato EF-1α gene served as the reference gene. Each qRT-PCR assay was repeated with three biological samples. The relative expression levels of genes were calculated using the 2− △△Ct method.

For the mature miRNA159 expression analysis, the cDNA was generated through reverse transcription from the total RNA by using the miRcute miRNA First-Strand cDNA Synthesis Kit (TIANGEN, Beijing, China). The qRT-PCR analysis was performed using an miRNA qPCR Detection Kit (SYBR Green) (TIANGEN). The tomato U6 small nuclear RNA gene was used as an internal control. The gene-specific primers used in these procedures are listed in Supplementary Table S4.

In situ hybridization

Developing tomato flower buds at various developmental stages were collected, fixed, embedded, sectioned, and subjected to in situ hybridization as described previously. The sense and antisense probes for SIMYB33 were generated using SP6 or T7 RNA polymerase, respectively. The primers used for the synthesis of the probes are listed in Supplementary Table S4.

Construction of the RNAi vector and tomato transformation

A 496-bp fragment of the SIMYB33 coding sequence was amplified to produce the sense strand using primers containing Spe I and BamH I sites and the antisense strand using primers containing Asc I and Swa I sites. The two amplified fragments were cloned into the pFGC1008 vector in the reverse orientation. Then, the resulting SIMYB33-RNAi vector was transformed into tomato as described by Chen et al. The positive plants were verified through PCR testing. After self-crossing, the seeds of the T0 generation were harvested and then sown in the soil matrix together with those of the WT. The seedlings of the T1 lines and WT were cultured in a growth room under the same environmental conditions. To avoid the impact of external factors such as tissue culture procedures and antibiotics on phenotypic identification, the positive transformants of the T1 generation were selected by PCR examination rather than by antibiotic screening. The specific primers used for RNAi construct generation and the identification of transformants are listed in Supplementary Table S4.

Pollen germination

Pollen germination tests were performed according to Carrera et al. Briefly, pollen grains were collected and deposited on glass slides with germination medium (pH 5.8) containing 0.292 M sucrose, 1.27 mM Ca(NO3)2, 1.62 mM H3BO3, 1 mM KH2PO4, and 0.6% agarose. After
incubation at 25°C in the dark for 2 h, the germinated pollen grains were observed and counted using a microscope.

**Scanning electron microscopy (SEM)**

The mature anthers were collected and fixed overnight in 4% glutaraldehyde at 4°C and washed with 0.1 M phosphate-buffered saline (PBS, pH 6.8) four times. The samples were dehydrated through an ethanol series (10%, 30%, 50%, 70%, 80%, 90%, and 100%), critical point dried using a desiccator, and coated with gold–palladium in an ion coater (Eiko IB5, Tokyo, Japan). Digital images of pollen morphology were observed using a scanning electron microscope (Hitachi S-4800, Tokyo, Japan) with an accelerating voltage of 2 kV.

**Histology**

Samples of flower buds containing various developmental stages of pollen were fixed, embedded, transversely sectioned (5-μm thick), dewaxed, and stained as previously described. The sections were stained with 1% sarranine and a fold change > 2. The KEGG (Kyoto Encyclopedia of Genes and Genomes) classification of DGEs was conducted with KOBAS 2.0 software.

**Transcriptome analysis**

Total RNAs from the shoot apices and anthers at specified stages were extracted with an RNA kit (TaKaRa) and used for transcriptome analysis. Digital gene expression (DGE) libraries were constructed as described previously. Three biological replicates were included in each library. RNA sequencing was performed on the Illumina HiSeq 4000 platform using paired-end technology by Majorbio Corporation (Shanghai, China). Bioinformatics analysis of the DGE data was carried out using a scanning electron microscope (Hitachi S-4800, Tokyo, Japan) with an accelerating voltage of 2 kV.

**Analysis of the promoters of target genes**

The 2-kb promoter sequences (before ATG) of the target genes were acquired, and the cis-acting elements were analyzed using the online database PlantCARE (http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html/).

**Determination of sucrose, glucose, and fructose contents**

Anthers at the mature pollen stage were collected and immediately frozen in liquid nitrogen. The concentrations of sucrose, glucose, and fructose were measured by HPLC (Shimadzu LC-30A, Kyoto, Japan) following Chen's method, and sucrose (CAS: 57-50-1), D-(+)-glucose (CAS: 50-99-7), and D-(-)-fructose (CAS: 57-48-7, Sigma, USA) were used as the corresponding standards.

**Statistical analysis**

Data analyses were performed using Excel 2010. The significance of differences between the control and experiment groups was assessed using two-tailed Student's t tests with SPSS (Statistical Product and Service Solutions) 23.0. The threshold values corresponding to P < 0.05 and P < 0.01 were indicated as * and **, respectively.

**Accession numbers**

The accession numbers of the GAMYB orthologs from various species used in this study are as follows: Arabidopsis MYB33 (At5g06100), MYB65 (At3g11440), MYB101 (At2g32460), Capsicum annuum GAMYB1 (CA01g13490), GA MYB2 (CA06g22230), Coffea canephora MYB65 (Cc 01_g07440), Cucumis melo GAMYB (XP_008456639), Cucumis sativus GAMYB1 (Csa009014), GAMYB2 (Csa 019830), GAMYB3 (Csa013555), Glycine max GAMYB1 (H M447241), GAMYB2 (HM447242), Gossypium raimondii GAMYB-like (Gorai.009G301100), Hordeum vulgare GA MYB (AA2G2863), Lolium temulentum GAMYB (AAD 31395), Malus domestica GAMYB-like (MDP0000147309), Manihot esculenta GAMYB-like (XP_021598631), Oryza sativa GAMYB (CAA67000), Petunia hybrida MYB33a (Peaxi162Scf00526g00820), MYB33b (Peaxi162Scf00342g00113), Populus trichocarpa GAMYB (XP_002313492), Prunus persica GAMYB (Prupe.2G050100), Ricinus communis GAMYB (XP_015577302), Siganus rivulatus GAMYB (Sopen06g22230), Solanum pennellii GAMYB (Sopen01g004560), SOLYD-2a GAMYB-like (Sopen06g030050), Solanum tuberosum GAMYB1 (PGSC0003DMG400022689), Solanum tuberosum GAMYB2 (PGSC0003DMG400005918), Sorghum bicolor GAMYB (Sobic.003G331100), Vitis vinifera GAMYB (XP_010651548), and Zea mays GAMYB (AIW47221).

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**Author contributions**

Y.Z. and Y.L. designed the experiments, Y.Z., B.Z., TY., J.Z., B.L., and X.Z. performed the experiments. Y.Z., B.Z., and TY. analyzed the data. Y.Z. wrote the paper. All authors reviewed and approved the paper.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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