Comparative transcriptomic analysis of early fruit development in eggplant (Solanum melongena L.) and functional characterization of SmOVATE5

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Abstract

Eggplant, a solanaceous crop that has undergone a long period of domestication, is one of the most important vegetables worldwide. The shape of its fruit is an important agronomic trait and consumers in different regions have different preferences. However, a limited understanding of the molecular mechanisms regulating fruit development and shape has hindered eggplant breeding. In this study, we performed morphological observations and transcriptome analysis of long- and round-fruited eggplant genotypes to understand the molecular regulation during the early development of different fruit shapes. Morphological studies revealed that the two varieties already exhibited distinctly different phenotypes at the initial stage of fruit development before flowering, with rapid fruit enlargement beginning on the sixth day after flowering. Comparative transcriptome analysis identified phytohormone-related genes that were significantly upregulated on the day of flowering, indicating they may be involved in regulating the initial stages of fruit development. Notably, *SmARF1* showed a sustained upregulation pattern in both varieties, suggesting that it may promote eggplant fruit growth. In addition, several differentially expressed genes of the SUN, YABBY, and OVATE families are potentially involved in the regulation of fruit development or fruit shape. We demonstrated that the *SmOVATE5* gene has a negative regulatory function suppressing plant growth and development. In conclusion, this study provides new insights into the molecular regulatory mechanisms of eggplant fruit development, and the genes identified may provide valuable references for different fruit shape breeding programs.

Key Message

Comparative transcriptome analysis of early fruits of long and round eggplants, SmOVATE5 is involved in regulating fruit development.

1. Introduction

Most fruit develops from the ovaries or other parts of the flower in angiosperms upon pollination of the pistil. Fruit development is closely related to the mechanisms of cell division, growth, and differentiation. To some extent, the final fruit shape is the result of a defined number of cell divisions that occur within the developing fruit after fertilization (Gillaspy et al. 1993) (Dinneny et al. 2005). The study of the genetic foundation and regulatory mechanisms of fruit shape is critical for accelerating the quality breeding process of vegetable crops.

According to Asa Gray’s classification (Hagemann 1990), eggplants can be classified as single-fruited fleshy fruits whose fruit development process is similar to that of tomatoes and can be roughly divided into four stages. The first and most decisive is the stage of development of the floral meristem (Suzaki et al. 2004; Taguchi-Shiobara et al. 2001; J et al. 1992; Clark et al. 1993). Next, the ovary grows along three axes after fertilization: the proximal-distal, medial-lateral, and abaxial-adaxial axes. The length and width of the fruit are determined by the degree of growth along the proximal-distal and medial-lateral axes, respectively. The degree of cell division on distinct growth axes results in different fruit morphologies

(Girin et al. 2009). Thus, early fruit development directly determines the final fruit length and diameter (Jiang et al. 2015).

Many factors can affect the early fruit development process. Phytohormones such as auxin and gibberellic acid (GA) are the primary regulators that control fruit expansion in different plant species. Phytohormones are produced in the developing seeds and are critical for early fruit development (Ozga and Reinecke 2003). The regulation of gene expression by auxin is directly controlled by members of the auxin response factor (ARF) family of transcription factors (Mockaitis and Estelle 2008). It has been reported, that ARF8 negatively regulates fruit initiation and growth in Arabidopsis (Goetz et al. 2006). Signaling by GA is negatively regulated by the main repressor DELLA protein, which interrupts transcription by directly binding to transcription factors such as PIF4, encoded by PHYTOCHROME INTERACTING FACTOR 4 (Daviere and Achard 2016). Auxin is known to facilitate GA biosynthesis in many plant species. Indeed, in tomato fruit, the expression of GA biosynthetic genes (GA20ox and/or GA3ox) is promoted by auxin treatment (Frigerio et al. 2006).

Genes are decisive factors in controlling phenotypes. Shape is an important criterion to evaluate the commodity quality of fruits and this has been studied extensively in tomatoes. Genes that have been reported to control fruit length in tomatoes include *OVATE* and *SUN*. In 1999, Grandillo et al. mapped a major gene controlling the oval shape of tomatoes on chromosome 2 using the population genetics approach (Grandillo et al. 1999). The gene sequence was determined by Ku et al. based on fine mapping and they subsequently reported that this gene caused tomato fruit to change from round to pear-shaped due to the advance of the terminator on the second exon (Ku et al. 2001). The *Sun* loci have been located on chromosome 7 and were shown to play a role in regulating fruit length following pollination (Knaap and Tanksley 2001). Later, it was found that the *SUN* gene encoded a member of the IQ67 domain family through location-based cloning and its function of controlling fruit length was verified through transgenic and gene interference methods (Xiao et al. 2008). Overexpression of the *SUN* gene leads to a dramatic increase in tomato ovary length, which in turn leads to longer fruit (Wu et al. 2011). In 2008, Cong et al. cloned the *FAS* gene encoding FAS, a member of the YABBY gene family, at the *fasciated* loci by fine localization. Due to the insertion of a large fragment of 6–8 kb in the first intron, the gene expression level is reduced, which leads to an increase in ventricular number in cultivated varieties (Cong et al. 2008). In conclusion, the current research on tomato fruit type regulation has been very in-depth and key genes have been identified. However, the research on eggplant fruit shape is relatively weak.

Eggplant (*Solanum melongena L.*), a member of the Solanaceae family, is a good source of minerals, vitamins, and certain polyphenols that show potent antioxidant activity (Nisha et al. 2009; Sudheesh et al. 1999). In eggplants, fruits are derived from the ovaries and display a large diversity in morphology. Their shapes range from round to club-shaped, short rod-like, and elongated. This attribute is often measured as the fruit height to width ratio and coined as the ‘fruit shape index’ (Gonzalo and van der Knaap 2008). Consumers from different regions display great differences in their preference for eggplant fruit shape (Yong et al. 2006). One of the basic goals of eggplant breeding is to select a commercial fruit shape that is in line with consumers' wishes in the selling area. In this study, we used fruits obtained at -6, 0, and 6

days after anthesis (DAA) as experimental materials for RNA-Seq data to compare the transcriptomes of early eggplant fruits with two different shapes. Subsequently, we analyzed differential gene expression in the fruits of these three periods separately. Using pairwise comparative analysis we identified candidate genes that may be involved in regulating fruit development in the early stages of growth and that may influence the different shapes of the fruit. This work provides essential insights into the molecular network of eggplant fruit development.

2. Materials And Methods

2.1 Plant materials Preparation

The plant materials used in this experiment were the ‘Feng Shou’ (FS) and ‘Hua Min’ (HM) eggplants, which are stable local varieties cultivated by the Shanghai Academy of Agricultural Sciences. Plants of the two varieties were cultivated at the horticultural farm of Shanghai Jiao Tong University, China, in 2020 and 2021. The length and diameter of fruits from 10 individual plants of each variety were measured at maturity, their ratios were calculated, and the average was taken as the fruit shape index. Fruits were collected for transcriptome sequencing 6 days before anthesis, the day of anthesis, and 6 days after anthesis. Three-to-five fruits from different plants were pooled together as one biological sample for each eggplant variety. Three biological replicates were set for each group. Samples were immediately frozen in liquid nitrogen and stored at -80℃ until further use.

2.2 RNA Extraction and Sequencing

Total RNA was extracted from fruits using the Steady Pure Plant RNA Extraction Kit (Accurate Biotechnology (Hunan) Co., Ltd., China) according to the manufacturer’s instructions. Then, tested, high-quality RNA samples were constructed in RNA-seq libraries and sequenced using Illumina NovaSeq 6000 (2 × 150 bp read length) for transcriptome sequencing (Majorbio, Shanghai, China). The raw reads were initially filtered to obtain clean reads by removing low-quality sequences and adapters using SeqPrep software (https://github.com/jstjohn/SeqPrep). Clean reads were then mapped to the eggplant reference genome (http://eggplant-hq.cn/) by HISAT2 software (Kim et al. 2015) and assembled (Pertea et al. 2015). The quality was evaluated by saturation analysis. Duplicate reads and gene coverage analysis indicated that the RNA-Seq data were appropriate for subsequent analysis.

Genes that were differentially expressed between different developmental stages of the same varieties and between different varieties at the same developmental stage were screened using the evaluation criteria of |log2FC | > 1 and P-adjust < 0.05 (Love et al. 2014) and transcripts per million(TPM) reads were used to compare differentially expressed genes (DEGs) in two samples. A total of six cDNA libraries were constructed and sequenced with three biological replicates for each sample. The RNA sequence data set are available in the repository of NCBI Sequence Read Archive (SRA) under the GenBank accession BioProject: PRJNA851190 and accession numbers SAMN29213192 - SAMN 29213209.

2.3 Temporal analysis

A temporal analysis of the DEGs of each variety was done using Short Time-series Expression Miner (STEM) software with default parameters to identify trends in gene expression changes during eggplant fruit development (Ernst and Bar-Joseph 2006). Genes were analyzed by clustering according to the corresponding P-adjust, and genes with P-adjust < 0.05 were considered to be differentially expressed. These DEGs were enriched for gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using hypergeometric distribution tests. GO terms and KEGG pathways with P-adjust < 0.05 were considered functional annotations.

2.4 GO Term and KEGG Pathway Enrichment

The GO annotation analysis of the unigenes was performed using the high-score BLAST matches in the Swiss-Prot and TrEMBL Protein Databases (E < 1.0E-5) using Blast2GO (http://www.blast2go.com) (Conesa et al. 2005); Research resource identifier (RRID): SCR_005828). The unigenes were further classified using GO Slim http://www.geneontology.org/GO.slims.shtml). To assign the detected unigenes to biological pathways, KEGG pathway annotation was conducted using the online KEGG Automatic Annotation Server (KAAS, http://www.genome.jp/kegg/kaas/). The DEGs were analyzed for category enrichment and KEGG pathway enrichment using AgriGO (Du et al. 2010) using Fisher's Exact Test and FDR correction, respectively.

2.5 Validation of RNA-Seq Data by qRT-PCR

To validate the gene expression profiles identified by RNA-Seq, 10 genes with significant differential expression were selected for qRT-PCR validation. First, RNA (1 μg) was reverse transcribed in a volume of 20-μL using the *Evo M-MLV* RT Kit (Accurate Biotechnology (Hunan)), according to the manufacturer's instructions. Next, qRT-PCR was performed on a LightCycler 96 (Roche, Basel, Switzerland) utilizing the SYBR Premix Ex Taq II Kit (Takara, Kyoto, Japan). The PCR procedure was set as follows: 3 min at 95 ℃, followed by 40 cycles of 95℃ for 5 s, 60 ℃ for 30 s, and 72 ℃ for 30 s. Each sample was represented by three technical replicates and three biological replicates. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The *actin* gene (GU984779.1) was used as a constitutive control (Liu et al. 2012). All gene-specific primers used in this study are listed in Supplementary Table S7.

2.6 Overexpression of the *SmOVATE5* genes in Arabidopsis

To produce transgenic plants in which *SmOVATE5* was expressed under the control of the 35S promoter, the coding sequence (CDS) of *SmOVATE5* was cloned into the PHB-YFP vector to generate the 35S::SmOVATE5 recombinant plasmid. The Agrobacterium tumefaciens strain GV3101 carrying the recombinant plasmid was used to transform Arabidopsis Col-0 plants using the floral dip method (He et al. 2021).

2.7 Paraffin sections

Fruits of FS and HM eggplant were collected at -6, 0 and 6 days after pollination and fixed in FAA (70 percent ethanol, acetic acid, formaldehyde 90:5:5 v/v) for 24 hours at room temperature. Longitudinal

and transverse sections were stained with hematoxylin-eosin and scanned using a panoramic section scanner (PANNORAMIC DESK/MIDI/250/1000) after trimming, dehydration, embedding, and sectioning. The target area of the tissue was chosen for 400x imaging using CaseViewer 2.4 scanning software, and following imaging, cell area and number were measured and statistically analyzed using Image-Pro Plus 6.0 analysis software, with at least three biological replicates at each time point.

2.8 Statistical Analysis

All experimental data are expressed as the mean ± standard error. Variance analyses were performed using SPSS 17.0 (SPSS Inc, Chicago, IL, USA). P-values of < 0.05 were considered statistically significant.

3. Result

3.1 Morphological analysis of two eggplant species with different shapes

Given that early fruit development directly determines the final fruit length and diameter, we measured the length and diameter of FS and HM eggplant fruits from 6 days before anthesis to 8 days after anthesis (Fig. 1A). We found that the fruit shape index of FS was larger than that of the HM eggplant. This difference was evident 6 days before anthesis and was present during subsequent developmental stages. The final fruit shape index of FS (about 10) was almost ten times larger than that of HM (about 1) (t-test, $P < 0.05$), conferring different shape phenotypes (Fig. 1E). From 6 days before flowering to the day of flowering, the length and diameter of the fruits of both FS and HM did not change significantly (Fig. 1B, C). Subsequently, the fruit length of FS began to increase exponentially, and the fruit diameter also increased, but less than the length, resulting in an increase in the fruit shape index. However, the fruit diameter and length of HM increased at a similar speed, and while the fruit was significantly larger on the sixth day after anthesis the fruit shape index remained almost unchanged (Fig. 1D, E).

Because the shape and size of the organ are directly related to the number and size of cells (Pan et al. 2020), we next examined the fruit cytoarchitecture of FS and HM using paraffin sections (Fig. 1F). The longitudinal section data revealed a decrease in cell number in both FS and HM, however, the decline was greater in FS. In the first two periods, the cell sizes of the two varieties did not differ considerably, but by the sixth day after flowering, the cells of FS were significantly larger than those of HM (Fig. 1G). These findings imply that the elongated fruit of FS is caused by the enlarged longitudinal cells. Transversal section results revealed that the cell number of HM was significantly greater than that of FS and decreased more on the sixth day after flowering, whereas the cell size of both varieties did not change significantly from − 6 DAA to 0 DAA and increased significantly by the sixth day after flowering, and was more pronounced in HM than in FS (Fig. 1G), indicating that the flattened fruit of HM was caused by a greater number of transverse cells.

3.2 RNA-Seq Results

The fruit length and diameter of FS and HM changed considerably between the time points 0 and 6 DAA, with fruits showing a significant enlargement at 6 DAA compared with the previous periods (Fig. 1). Additionally, we assessed the regulation of gene expression before anthesis. To elucidate gene expression changes in FS and HM eggplant fruits during early development, we selected − 6, 0, and 6 DAA fruits for comparative analysis by Illumina sequencing. Three biological replicates were evaluated at each time point. A total of 18 cDNA libraries of early developing fruits were constructed for transcriptome sequencing. After removing low-quality reads, approximately 123.4 Gb clean data were obtained, and every sample was represented by over 6.05 clean data. We detected a total of 28992 expressed genes in eggplant fruit samples. The error rate of each sample was about 0.02%, i.e., below 0.1%, and the Q30 scores of clean bases were approximately 94% (Supplementary Table 1). The expressed genes were then utilized to generate a correlation matrix, to compare the similarity of all transcriptomes. The heat map revealed that each group's three biological repetitions are inextricably linked (Supplementary Fig. 1). Due to the high quality of the sequencing results, most reads could be mapped to the eggplant reference genome. The mapped ratio was about 88%-93% (Supplementary Table 1), and the quality was assessed by saturation analysis. In conclusion, the above outcomes indicated that the RNA-Seq sequencing results were reliable and could be further analyzed.

3.3 DEG Analysis

To identify genes involved in fruit development, a differential expression test was done between FS and HM eggplant cultivars at three developmental stages (-6, 0, and 6 DAA). Genes with adjusted p-values < 0.05 and absolute log2FC ≥ 1 were considered significant DEGs. A total of 2724 genes (Supplementary Table S2a-c) are shown in Fig. 2. There were 1082, 1100, and 1810 genes identified as DEGs at the three developmental stages between FS and HM eggplant (Fig. 2A). Of all DEGs, a total of 375 genes were found to be differentially expressed in all comparisons. A total of 362, 406, and 1063 genes were exclusively differentially expressed in the − 6, 0, and 6 DAA, respectively, during early fruit development. In addition, we also analyzed DEGs among different stages in the same cultivar and compared them with each to find some common DEGs. There were 1856 DEGs in FS1_vs_FS2 (-6 vs. 0 DAA), 2170 in FS2_vs_FS3 (0 vs 6 DAA), 3185 in FS1_vs_FS3 (-6 vs 6 DAA), 1962 in HM1_vs_HM2 (-6 vs. 0 DAA), 477 in HM2_vs_HM3 (0 vs. 6 DAA) and 2253 in HM1_vs_HM3 (-6 vs. 6 DAA) (Supplementary Table S2d-l; Fig. 2B, C). These findings showed that the expression differences within varieties were more obvious than among varieties.

3.4 Functional Annotation of DEGs

To further understand the function of these DEGs, GO term enrichment analysis (P < 0.05) was performed using all the annotated genes in eggplant as background. The top 20 terms enriched for DEGs of the three developmental periods between the two varieties are shown in Fig. 3. We found that DEGs in each period were significantly enriched into two major categories, i.e., biological processes and molecular function. The three biological processes with the most significant enrichment at 6 days before flowering were ‘gibberellin metabolic process’, ‘gibberellin biosynthetic process’, and ‘diterpenoid metabolic

process'. The GO terms related to 'pectin catabolic process', 'pectin metabolic process', and 'galacturonan metabolic process' were significantly enriched in the FS vs. HM on the day of flowering. For the third comparison (6 days after anthesis) between FS and HM eggplants, the three most enriched biological processes were 'microtubule-based movement', 'auxin-activated signaling pathway', and 'movement of a cell or subcellular component' (Supplementary Table S3). These processes are related to plant growth and development, indicating that there are great differences in the development of fruits of different shapes. Functional annotation analysis of DEGs in the same variety at different periods before and after anthesis is helpful to understand which genes play a greater role in early vs. late fruit development. For FS eggplants, most enriched GO terms between − 6 and 0 DAA were involved in biological processes, including 'beta-glucan metabolic process', 'polysaccharide catabolic process', and 'cellulose catabolic process', whereas for HM eggplants, the most significantly enriched GO terms between − 6 and 0 DAA were 'pectin catabolic process', 'cell wall biogenesis' and 'steroid metabolic process'. For the second comparison (0 DAA_vs_6 DAA), the DEGs in FS eggplants were found to be significantly enriched in the processes of 'microtubule-based movement', 'regulation of mitotic cell cycle' and 'regulation of cell cycle process' whereas for HM the three most enriched biological processes were 'meiosis ', 'meiosis cell cycle process' and 'male meiosis ' (Supplementary Figure S2, S5).

3.5 Comparison of trends in temporal gene expression during early eggplant fruits

To better understand the changes in gene expression throughout early fruit development, we used the STEM algorithm to group 4467 DEGs from FS eggplants and 2919 DEGs from HM eggplants into 16 profiles. Among these, 2814 FS DEGs were strongly clustered into five key files: two downregulated profiles (profiles 0 and 3) and three upregulated profiles (profiles 4, 6, and 7). Similarly, 2094 HM DEGs were classified into four profiles: two upregulated patterns (profiles 6 and 7) and two downregulated patterns (profiles 0 and 1). The DEGs in downregulated patterns may be involved in regulating fruit development before flowering, and up-regulated genes are likely to be involved in early fruit development after flowering. The DEGs in profiles 0, 6, and 7 were significantly enriched in both the FS and HM (Fig. 4). Most gene expression patterns during early fruit development were similar between FS and HM, but the main difference lay in the function of key genes therein.

To systematically investigate the biological functions of candidate genes, we extracted DEGs from profiles 0, 6, and 7 for further GO term and KEGG pathway analysis. In FS eggplants, GO analysis revealed 22 biological processes that were significantly enriched by the DEGs assigned to profile 0, and multiple metabolic processes were involved. The most enriched biological process was the 'lignin catabolic process'. The DEGs in profile 6 were firmly categorized into two molecular processes including 27 Biological processes and 17 Molecular functions. Genes within profile 7 were enriched mainly in the 'auxin-activated signaling pathway' (GO:0009734), 'rRNA metabolic process' (GO:0016072), and 'hormone-mediated signaling pathway' (GO:0009755) in biological processes (Supplement Figure S2). The GO terms with the highest representation for the HM profile groups are shown in Supplement Figure

S3. Among the biological progress categories, ‘phenylpropanoid metabolic process’ (GO:0009698), ‘polysaccharide catabolic process’ (GO:0000272), and ‘cell wall organization’ (GO:0071555), were the most significantly enriched functions in profiles 0, 6 and 7, respectively.

The KEGG pathway enrichment analysis was performed on the DEGs in the profiles using the R script, and when the P adjust < 0.05, the KEGG pathway function was considered to be significantly enriched. In total, we identified eight KEGG pathways, including ‘Zeatin biosynthesis’, ‘Pentose and glucuronate interconversions’, ‘Diterpenoid biosynthesis’, ‘Phenylpropanoid biosynthesis’, ‘MAPK signaling pathway – plant’, ‘Plant hormone signal transduction’, ‘Glutathione metabolism’, and ‘Ribosome biogenesis in eukaryotes’, that were enriched in profiles 0, 6 and 7 of FS and HM (Supplement Figure S4). Among these, ‘Plant hormone signal transduction’ was the most enriched in DEGs and was significantly enriched in profiles 6 and 7 of FS, and profile 6 in HM. These results indicated that most DEGs regulated during early fruit development appear to be associated with the functioning of metabolic pathways.

3.6 Analysis of plant hormone signaling pathway during eggplant early fruit development

Early fruit development in horticultural plants is notably dependent on hormonal control of cell division and expansion. Auxin encourages cell proliferation and growth along with gibberellic acid (GA), which in turn controls fruit development and enlargement following fertilization (He and Yamamuro 2022). Based on the results of GO and KEGG enrichment analysis, we found significant enrichment of phytohormone-related processes in both FS and HM. For example, ‘gibberellin metabolic process’ and ‘auxin-activated signaling pathway’ GO terms were enriched into profiles 6 and 7 of FS eggplants, respectively (Supplementary Figure S3). ‘Plant hormone signal transduction’ was enriched into profiles 6 and 7 of FS and profile 6 of HM eggplants in the KEGG pathway enrichment (Supplementary Figure S5). In total, we identified 50 plant-hormone-related genes in FS and 24 plant-hormone-related genes in HM, 17 of which were common to both (Fig. 5A), and annotation information for these 17 genes is presented in Supplementary Table S5. Subsequently, we annotated and performed cluster analysis on these 17 genes. As shown in Fig. 5, Smechr0100790, encoding the auxin response factor SmARF1, was consistently upregulated in both FS and HM eggplants.

3.7 DEGs of SUN, OVATE and YABBY gene families related to early fruit development

Previously, SUN, OFP, and YABBY were established to be key genes that regulate fruit morphology in tomatoes and other fruits (Huang et al. 2013). Therefore, we first identified 31 SUN genes, 9 YABBY genes, and 22 OVATE genes from the reference eggplant genome. We then searched for these genes in the RNA-seq data and detected 8 SUN genes, 12 OVATE genes, and 4 YABBY genes with different expression levels in FS or HM eggplant (Supplementary Table S6). From the clustering analysis and functional annotation results, most of the genes that were highly expressed before flowering, such as *SmSUN4*, *SmYAB3*, and *SmOVATE5*, showed similar expression trends in FS and HM, indicating that these

genes may have a negative regulatory function during the early fruit development of eggplants (Fig. 6). The expression of these three genes was higher in HM than in FS eggplants. In addition, we identified several genes showing differential expression patterns between FS and HM during fruit development including *SmYAB2* and *SmOVATE10*.

3.8 Validation of RNA-seq results by qRT-PCR

To confirm the accuracy of the RNA-seq results, qRT-PCR was performed on the 7 genes with consistently up-regulated expression (Fig. 5) and the 3 genes in Fig. 6. A very strong correlation was found between the transcriptome sequencing and qPCR data (Fig. 7). Although there were some minor differences in expression levels, the expression trends were essentially the same, indicating that the data obtained from transcriptome sequencing in this study are plausible.

3.9 Ectopic overexpression of *SmOVATE5* negatively affects leaf and silique growth in Arabidopsis

Genes with high sequence similarity may perform comparable functions. The tomato OVATE gene has been reported to play an important negative regulatory role in plant development (Liu et al. 2002), and we found the highest homology between *SmOVATE5* and *SlOVATE* genes using phylogenetic analysis (Supplementary figure S6). To verify the function of the *SmOVATE5* gene, its full-length coding sequence was transferred into Arabidopsis under the control of the CaMV35S promoter (Fig. 8). Real-time PCR assay showed that *SmOVATE5* was highly expressed in the transgenic lines OE1, OE2, and OE3, compared with the wild-type plant (Fig. 8B). The leaves of the transgenic lines were oval in shape and smaller in size compared with the wild type leaves (Fig. 8A). In addition, the transgenic lines produced shorter plants (Fig. 8C), and the siliques of the transgenic lines were shorter than those of wild-type Arabidopsis (Fig. 8D, E). These results suggest that *SmOVATE5* functions as an inhibitor of cell elongation and thus negatively regulates fruit development.

Discussion

Eggplant fruits develop from the ovary and their final shape depends on cell division and expansion at the early stages of fruit development. In the present study, we found that the fruit shape index of the long-fruited FS eggplant at maturity is about 10.3, which is about 10 times higher than that of rounded HM eggplant, and this shape difference is obvious as early as 6 days before anthesis (Fig. 1). Morphological changes are usually associated with significant differences in gene expression levels (Jiang et al. 2015). To investigate the mechanisms of gene regulation in early fruit development that determine the shape of eggplant fruit we chose the early fruits (6 days before anthesis, anthesis day, and 6 days after anthesis) from both eggplant varieties for transcriptome analysis, and identified a total of 28992 expressed genes. The number of differentially expressed genes was found to be significantly greater in FS than in HM (Fig. 2), suggesting that FS fruits undergo more pronounced changes during development. In addition, the number of DEGs within FS and HM at different developmental stages were both greater than those

between the two varieties, indicating that the degree of variation within the three development stages was greater than that between varieties.

To identify the biological functions of these DEGs, all of the annotated genes in eggplant were utilized as a background and GO enrichment analysis was performed (Supplementary Table S3). Before anthesis, the DEGs between FS and HM eggplants were mainly enriched in the GO terms of gibberellin metabolic and biosynthetic process, on the day of anthesis, they were mainly enriched in pectin catabolic and metabolic process, and after anthesis, they were mainly enriched in microtubule-based movement and auxin-activated signaling pathway. These results demonstrate that differences in polysaccharide metabolism and cellular microtubule movement between FS and HM begin to manifest themselves early in fruit development. It has been shown that polysaccharides are the main components of plant cell walls and that hemicelluloses and pectin metabolism are involved in the biosynthesis and degradation of cell walls during early apple fruit development, thus contributing to a large extent to the formation of apple fruit texture (Dheilly et al. 2016). In previous studies, microtubule-related genes were found to play special functions in rapid cell division and expansion during early fruit development in cucumbers (Yang et al. 2013). Phytohormones coordinate multiple aspects of plant growth and development, including fruit initiation. Fruit initiation has traditionally been attributed to auxin, GA, and cytokinin (Gillaspy et al. 1993). Coincidentally, the cellulose catabolic process and pectin catabolic process were significantly enriched in FS and HM eggplant between -6 DAA and 0 DAA, which may indicate that plant polysaccharide metabolic pathways play a prominent role in mediating the initial stages of fruit development (M et al. 1977). From 0 DAA to 6 DAA, microtubule-based movement and meiosis II cell cycle process were detected as significantly enriched in FS and HM, respectively (Supplementary Figure S2), which indicated that the cells around the ovary divide and expand rapidly after pollination (Dreesen et al. 2012).

During early fruit development, we detected a total of 4467 and 2919 genes differentially expressed in FS and HM varieties, respectively, which were categorized into 16 profiles based on STEM analysis. Three profiles (0, 6, and 7) were obtained for both FS and HM, with profiles 0 being downregulated and profiles 6 and 7 upregulated. In FS eggplants, we identified several terms that were significantly enriched in upregulated DEGs, namely, ‘cell wall polysaccharide metabolic process’ and ‘auxin-activated signaling pathway’ (Supplementary Figure S3), while ‘polysaccharide metabolic process’ and ‘cell wall organization’ were most enriched in upregulated DEGs of HM eggplants (Supplementary Figure S4). These biological processes that are enriched in upregulated expression of genes have been reported previously and are thought to be particularly beneficial for fruit growth and development. The cell wall is composed of pectin, hemicellulose, and cellulose as well as some structural proteins (Dheilly et al. 2016). In addition to producing the strength required by the plant, the cell wall defines cell shape, cell size, and cell function (Marowa et al. 2016). Furthermore, cell wall structures that contribute to differences in softening rates in apple fruit are formed early in fruit development (Ng et al. 2013). The sucrose transporter Pu SUT and the β-glucanases Pu bglu1, Pu bglu2 and Pu bglu4 are highly expressed during the initial stages of fruit development (Xinyue et al. 2016), which is consistent with the results of the present study that suggest that several genes related to cell wall polysaccharide metabolism are indeed critical during the early stages of fruit development.

The regulation of fruit development by plant hormones has been previously reported. The use of these hormones, either alone or in combination, can stimulate fruit growth in a range of plant species (Ozga and Reinecke 2003; Ozga et al. 2002; Vivian-Smith and Koltunow 1999). Several lines of evidence have demonstrated that an auxin signal is generated after fertilization, which is considered to upregulate GA biosynthesis, which in turn activates GA signaling in the ovules, thereby promoting fruit growth (Mezzetti et al. 2004; Dorcey et al. 2009). In our study, the DEGs with upregulated expression were enriched for phytohormone-related pathways, for example, 'auxin-activated signaling pathway' (GO:0009734) and 'hormone-mediated signaling pathway' (GO:0009755) in biological processes, indicating important roles of plant hormones in fruit development. Among these hormones, it has been reported that auxin was first triggered after flower opening and promotes floral organ enlargement (Gillaspy et al. 1993). The auxin signal then promotes the biosynthesis of other plant hormones such as GA, and the interplay of auxin with other hormones thereby regulates fruit growth and development (Dorcey et al. 2009). We screened 17 hormone-related genes that were significantly enriched in both FS and HM among the upregulated genes. Expression clustering analysis (Fig. 5), revealed, that these were significantly activated on the day of flowering in both eggplant varieties. For example, the auxin response factor *SmARF1* was upregulated in both FS and HM. As key players in the auxin signaling pathway, ARFs regulate cell enlargement and plant growth by activating or repressing the expression of auxin-responsive genes-(Supplementary Figure S6). In plants, ARFs have been proven to mediate auxin signal transduction and to regulate growth. In melons, *CmARF1* expression was linked to fruit growth during early development (Wu et al. 2020). Furthermore, our results show that the expression of *SmPIF4* and *SmAUX22* was consistently upregulated in FS eggplants and, similarly, *SmIAA26-2*, *SmEBF2-1*, *SmEIL3*, and *SmERF1B-1* were also up-regulated in HM eggplants (Fig. 5), suggesting that these hormone-related genes play a role in promoting early fruit development since their expression is positively correlated with fruit growth. Conversely, differences in gene expression patterns between cultivars may result in alterations in cell division and expansion rates, resulting in varied fruit morphologies.

Several genes have been reported to be involved in the regulation of fruit shape during fruit development. Among these genes, FASCIATED (FAS), which belongs to the YABBY gene family, regulates fruit shape by affecting ovary numbers, while SUN and OVATE are key regulators controlling fruit elongation, all of which have been reported to affect blueberry fruit morphology during pre-flowering and post-pollination stages (Yang et al. 2018). The SUN protein harbors an IQD domain, which plays an important role in plant development processes (Cai et al. 2016). OVATE belongs to a plant-specific transcription factor family that plays a significant role in the growth and development of Arabidopsis and tomato (Zhang et al. 2020). In the present study, we screened for genes that are differentially expressed in FS and HM eggplants and found that most genes were expressed at higher levels before flowering (Fig. 6). Similarly, it has been reported that *SlOVATE* mRNA was detected only around the flowering time (Liu et al. 2002) and that OVATE represents a class of negative regulatory proteins important in plant development. To verify the molecular functions of the differential expressed genes that we identified in our study, we selected the *SmOVATE5* gene, which has the highest homology with the tomato *SlOVATE* gene, for functional validation in Arabidopsis-(Supplementary figure S6). Arabidopsis lines that were

overexpressing *SmOVATE5* had smaller and rounder leaves and shorter stalks and siliques than wild-type plants (Fig. 8). This result demonstrated that the *SmOVATE5* gene inhibits plant growth, in agreement with our transcriptome results. Differential expression of genes may lead to differences in traits. For example, slight changes in *CaOVATE* expression were sufficient to induce changes in pepper shape (Tsaballa et al. 2011). Our study identified *SmYAB2* and *SmOVATE10* as more highly expressed in HM than in FS. Therefore, these genes are potentially involved in the regulation of eggplant fruit shape, and in the future, they can serve as candidate genes for genetic transformation and phenotypic characterization to further investigate their role in fruit development.

Conclusion

In this study, we performed a comparative transcriptome analysis of two eggplant varieties with different fruit shapes (FS and HM) to characterize the genes and associated pathways that control early fruit development in differently shaped varieties. We identified several pathways that may contribute to the regulation of the shape and size of these fruits, including pathways related to phytohormone signaling, cell wall polysaccharide metabolic processes, and cell cycle regulation. In addition, we identified differentially expressed genes in the SUN, YABBY, and OVATE gene families and compared their expression patterns in two varieties, hypothesizing that these DEGs may have functions in regulating fruit development. We selected the *SmOVATE5* gene for functional validation and demonstrated its ability to negatively regulate fruit development. Several other candidate genes that may affect the final shape of fruit still need to be further investigated in depth for their potential molecular functions.

Declarations

Author contributions

SS, HG and HC conceived and designed the research. YL, HG and HC offered the experimental resources. SS, DL and SL performed experiments. SS analyzed data and wrote the manuscript. SS, YW, XT and YL performed data curation. All authors contributed to the enhancement of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

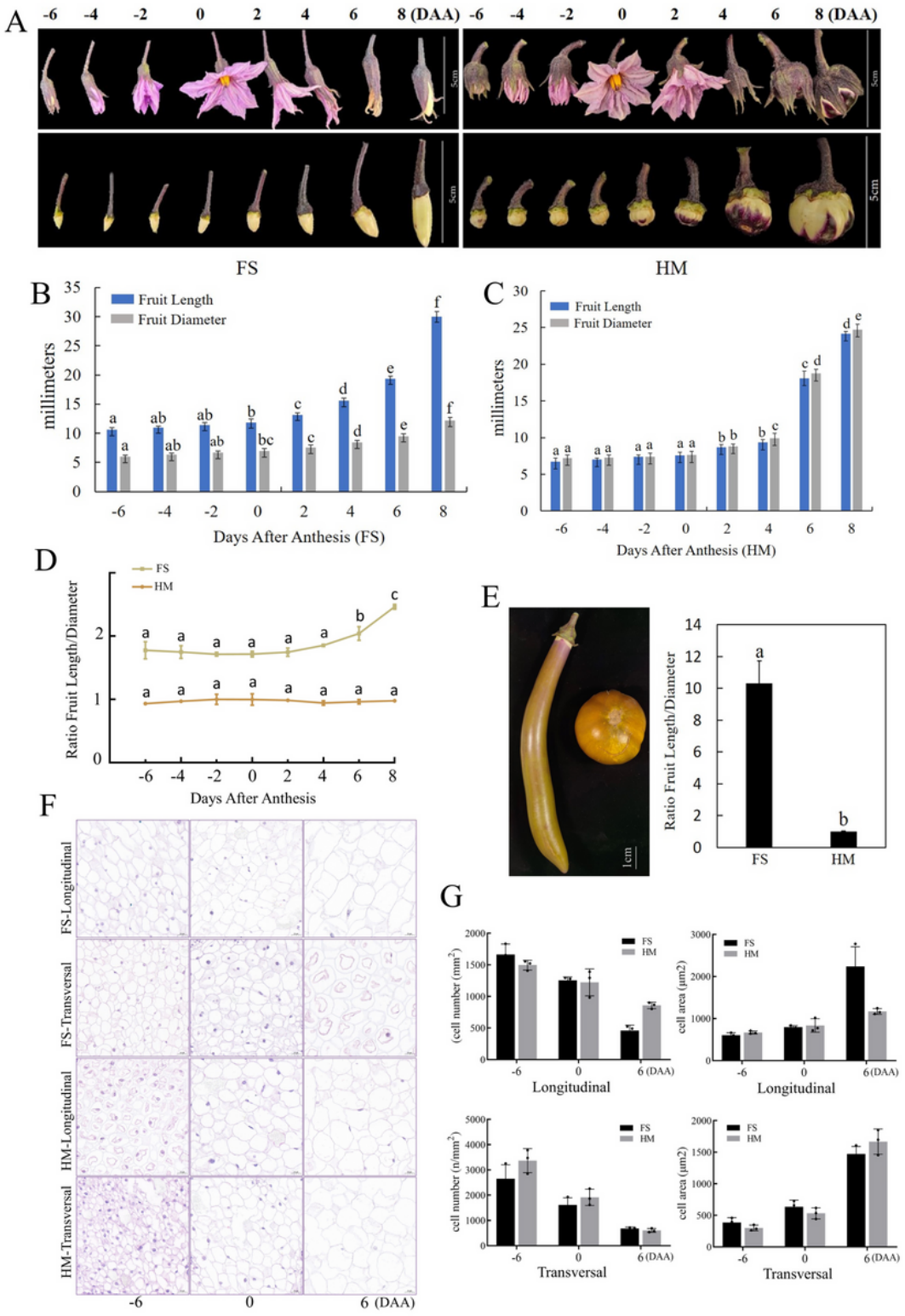

Figure 1

Morphological changes during early fruit development in two different cultivars of eggplant. (A) Fruit morphology of long-fruited (FS) and round-fruited (HM) eggplant from 6 days before flowering to 8 days after anthesis. (B) Fruit length and diameter of FS eggplants. (C) Fruit length and diameter of HM eggplants. (D) Fruit shape index of FS and HM fruits. (E) Morphology and fruit shape index during the fruit ripening stage. (F) The cellular structure of FS and HM fruits at different developmental stages,

including transverse and longitudinal sections. The scale bar is 20 μm. (G) Cell number and cell area of FS and HM fruits, as shown in (F). Different lowercase letters indicate statistical differences ($P < 0.05$) as determined by analysis of variance (ANOVA) using Duncan's New Multiple Range test method. DAA, days after anthesis

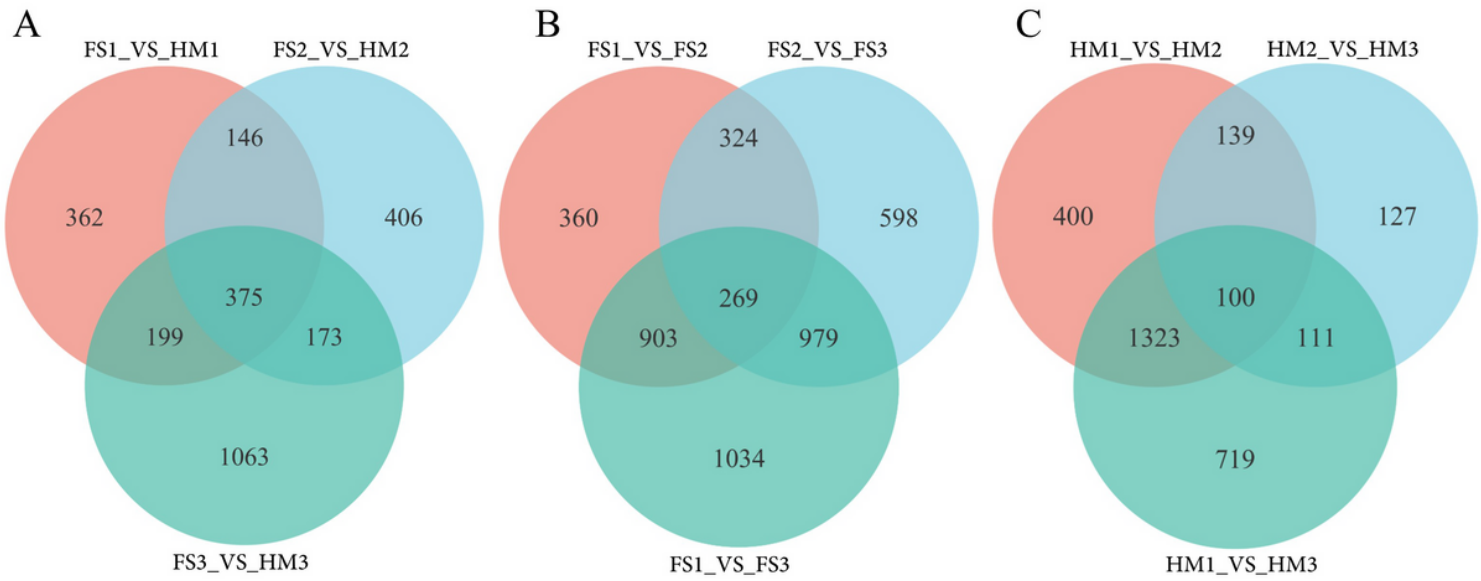

Figure 2

Venn diagram of the number of DEGs during pairwise comparisons of long-fruited (FS) and round-fruited (HM) eggplants. Comparison of DEGs in FS and HM during each of the three developmental stages (A). Comparison of DEGs during the three developmental stages of FS (B). Comparison of DEGs during the three developmental stages of HM (C)

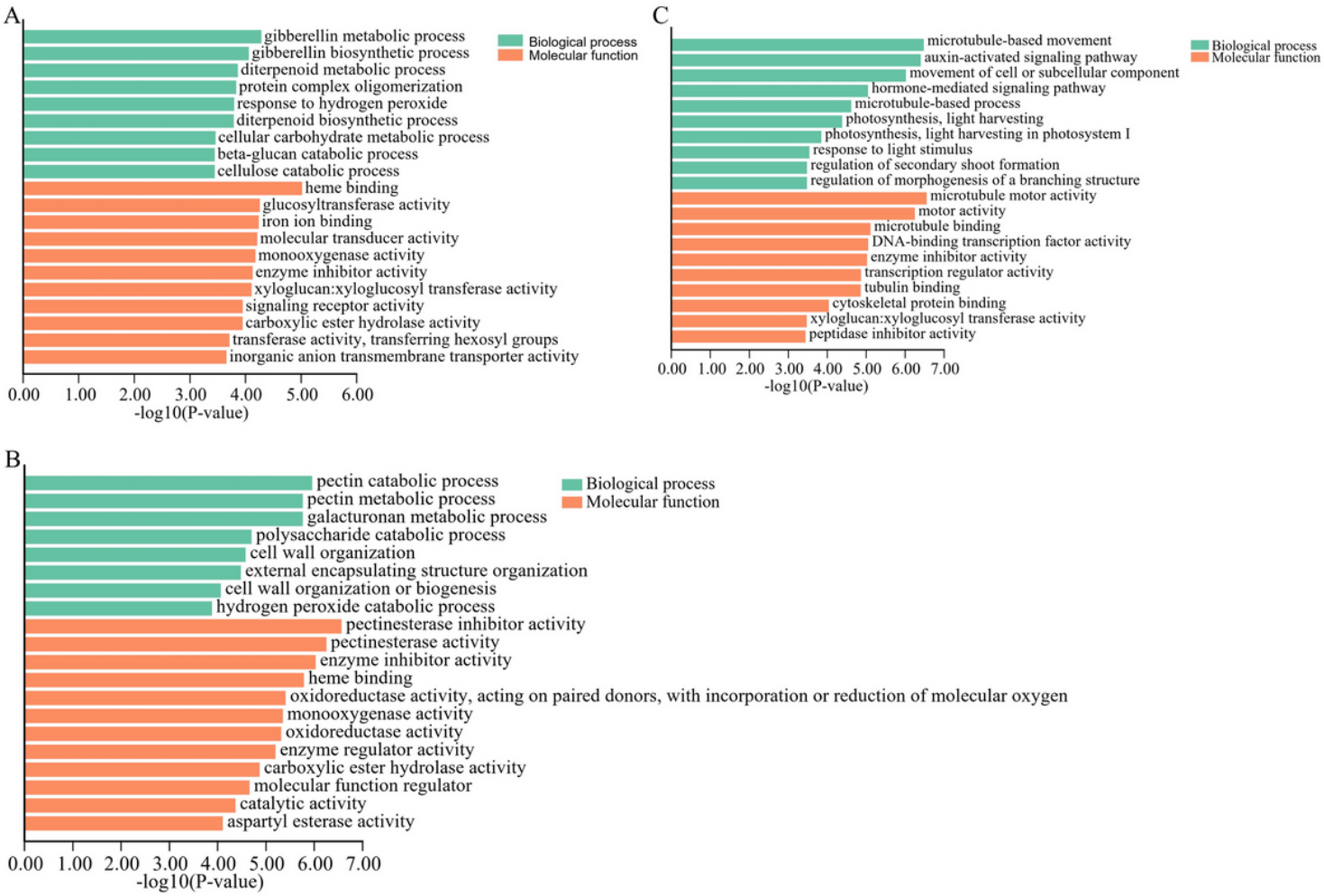

Figure 3

GO enrichment analysis of DEGs identified in the pairwise comparisons of FS and HM. (A) FS1_vs_HM1 (-6 DAA). (B) FS2_vs_HM2 (0 DAA). (C) FS3_vs_HM3 (6 DAA)

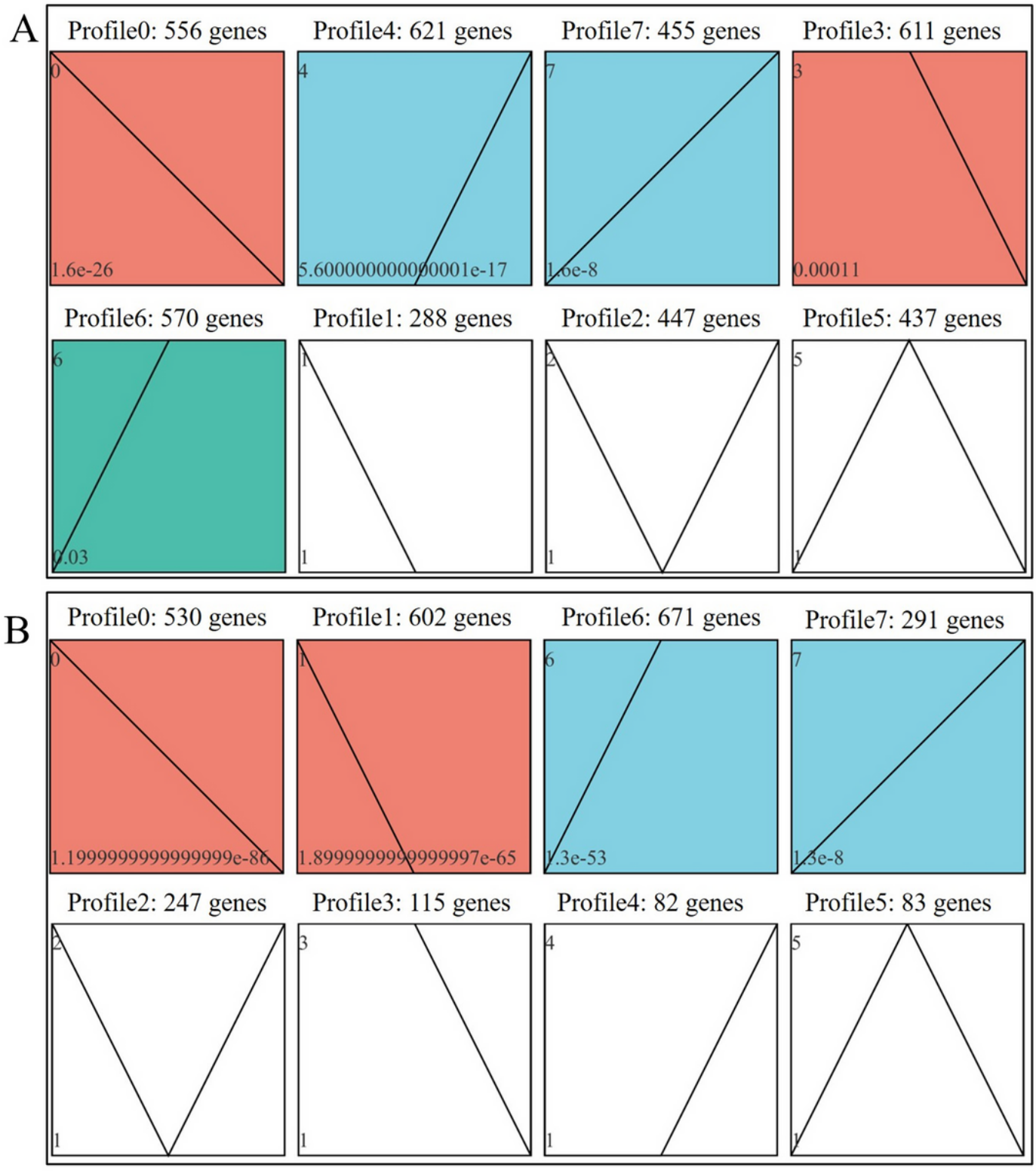

Figure 4

Patterns of gene expression across three developmental stages in FS (A) and HM (B) eggplants were inferred by Short Time-series Expression Miner (STEM) analysis. Each square represents a trend of gene expression. The text above each square indicates the profile ID number and the number of genes within that profile. The black line represents the expression tendency of all the genes. A colored square indicates that the pattern was significantly enriched (P values are listed in the bottom left corner of each

square). Squares of the same color represent similar trends. The profiles were ordered based on the degree of significance

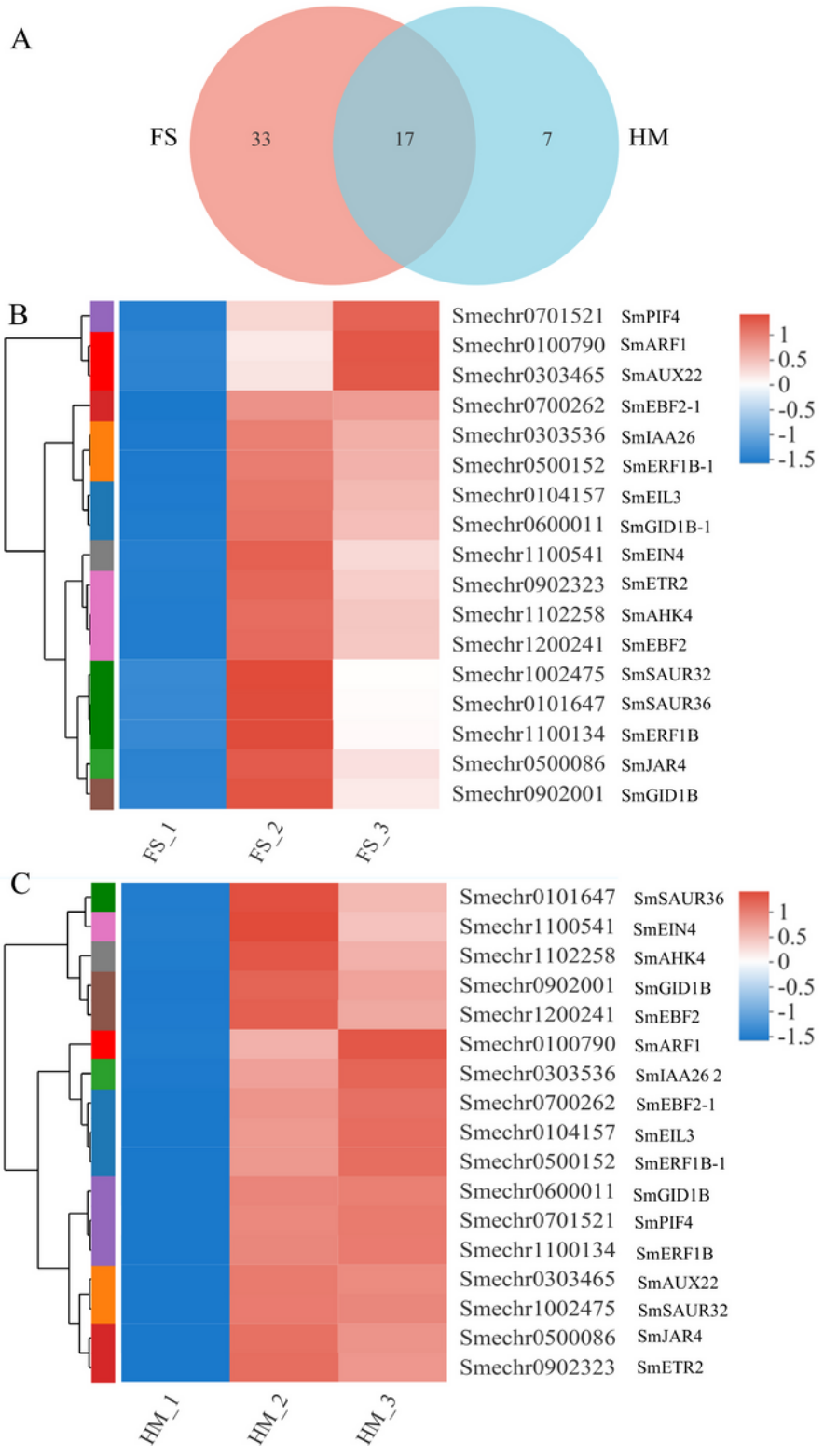

Figure 5

Trends in the changes in expression of key genes associated with plant hormones in two eggplant varieties. (A) Differentially expressed genes (DEGs) in the plant hormone signal pathway for FS and HM.

17 DEGs are common to both varieties. (B) DEGs for FS comparing -6 DAA (FS_1), 0 DAA (FS_2), and 6 DAA (FS_3). (C) DEGs for HM comparing -6 DAA (HM_1), 0 DAA (HM_2), and 6 DAA (HM_3)

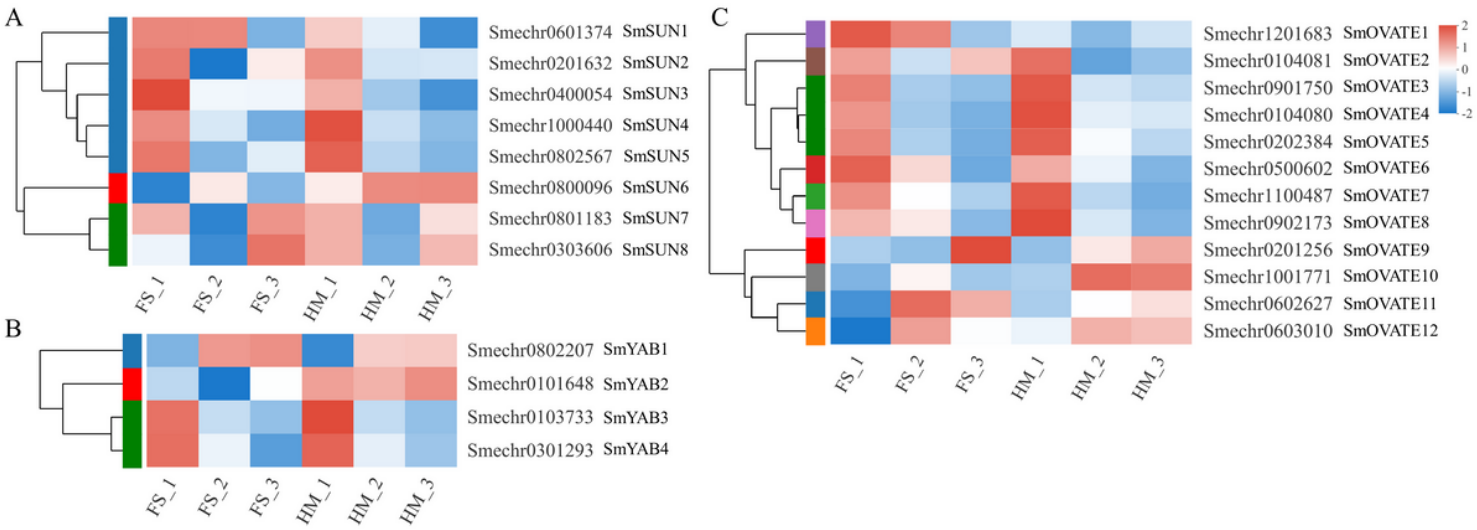

Figure 6

The expression level heatmap of selected DEGs from SUN, YABBY, and OVATE gene families in FS and HM eggplants. (A) SUN. (B) YABBY. (C) OVATE. -6 DAA (FS_1, HM_1), 0 DAA (FS_2, HM_2), and 6 DAA (FS_3, HM_3)

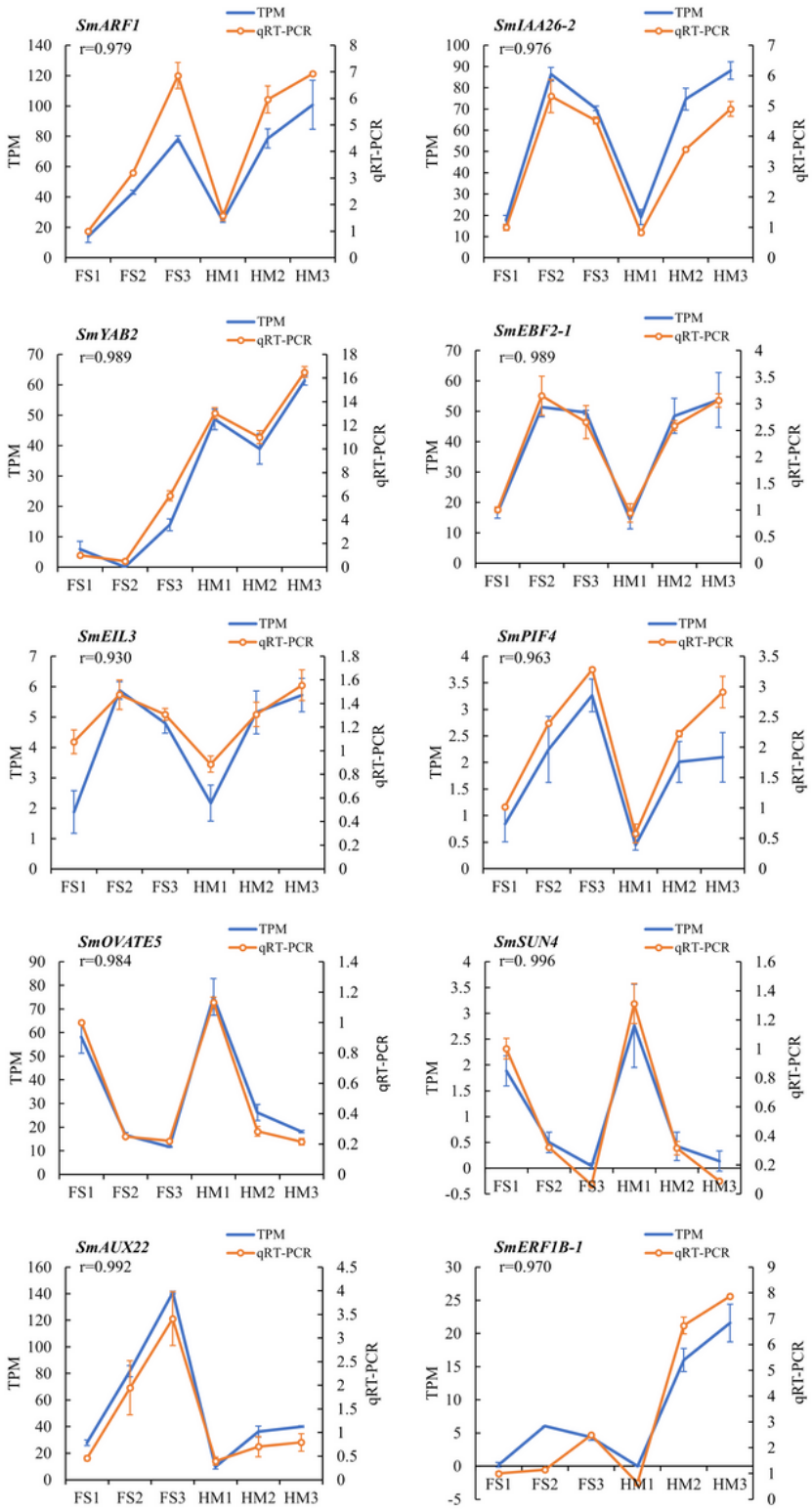

Figure 7

Validation by qRT-PCR of 10 DEGs that had been identified by RNA-seq. Correlation analysis showed the correlation between RNA-seq data and qRT-PCR was strong (Pearson R > 0.93). The left vertical axis indicates Transcripts Per Million reads (TPM; blue lines) and the right vertical axis refers to the quantitative real-time polymerase chain reaction (qRT-PCR; orange lines)

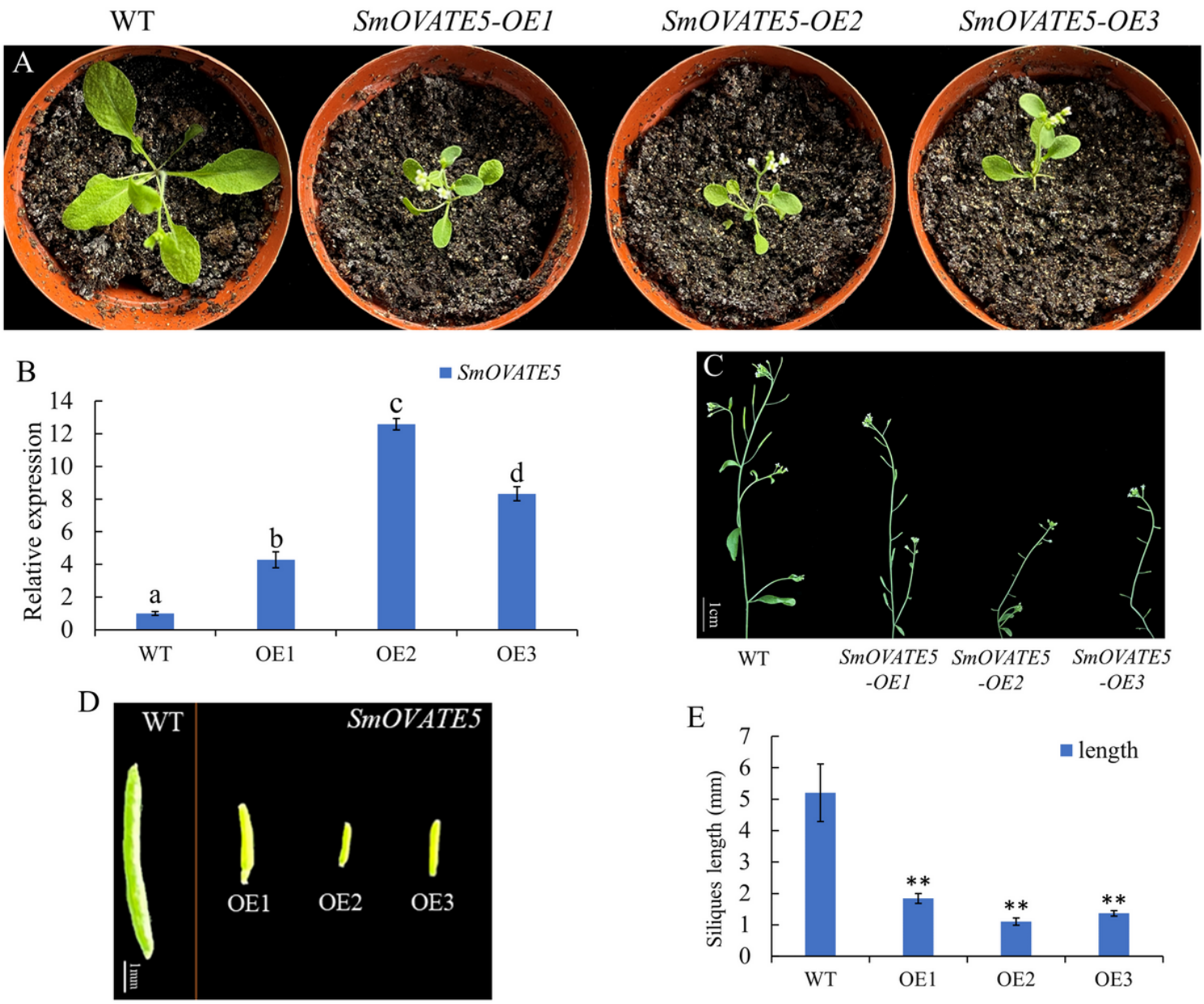

Figure 8

Functional analysis of the *SmOVATE5* gene. A) Seedlings of four-week-old Arabidopsis transgenic lines overexpressing *SmOVATE5* and the wild-type (Col-0). B) Expression of *SmOVATE5* in leaves of transgenic lines (OE1, OE2, and OE3) and wild type (WT). Error bars represent the SE of three biological replicates. Different lowercase letters indicate significant differences at $P < 0.01$ based on Fisher's LSD test. Plants (C) and siliques (D) of eight-week-old Arabidopsis transgenic lines overexpressing *SmOVATE5* and the wild type (Col-0). E, Comparison of the silique length between transgenic lines and the wild type. **, $P<0.01$ (Student's t-test)

Supplementary Files

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