Characterization of Genes Associated with TGA7 During the Floral Transition

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Abstract

Background

The TGA family has ten members and plays vital roles in plant defence and development in *Arabidopsis*. However, involvement of *TGAs* in control of flowering time remains largely unknown and requires further investigation.

Results

To study the role of *TGA7* during the floral transition, we first tested phenotypes of *tga7* mutant, which displayed delay-flowering phenotype under both long-day and short-day conditions. We then performed flowering genetic pathways analysis and found that both autonomous and thermosensory pathways may affect *TGA7* expression. Furthermore, to reveal differential gene expression profiles between wild-type (WT) and *tga7*, cDNA libraries were generated for WT and *tga7* mutant seedlings at 9 DAG (days after germination). For each library, deep-sequencing produced approximately 6.67 Gb of high-quality sequences with the majority (84.55%) of mRNAs between 500 and 3000 nucleotides in length. Three hundred and twenty-five differentially expressed genes (DEGs) were identified between WT and *tga7* mutant seedlings. Among them, four genes are associated with flowering time control. Differential expression of the four flowering-related DEGs was further validated by qRT-PCR.

Conclusions

Transciptomic sequencing coupled with flowering genetic pathways analysis provides a framework for further studying the role of *TGA7* in promoting flowering.

Background

TGACG-Binding (TGA) transcription factors (TFs) belong to the bZIP transcription factor family. There are ten members in the TGA family and they play essential roles in plant defence and development in *Arabidopsis* [1–3]. These TGAs can interact with *NPR1* (*non-repressor of pathogenesis-related gene 1*) gene, which involves in SA-mediated gene expression (such as *PR-1*) and disease-resistance [4, 5]. These TGAs can bind to *cis*-regulatory TGACG elements [6]. Studies show that this element is present in promoters of *PR1*, which are required for *PR1* gene expression in response to SA and interact with NPR1 [4, 7–9]. However, NPR1 cannot bind directly to *PR-1* promoter, but being recruited to the promoter by its physical interaction with TGAs to regulate the expression of *PR-1* [4, 6–9].

Among the ten *Arabidopsis* TGAs, NPR1 can interact with seven of them [7, 8, 10]. Those seven TGAs are further classified into three subclades - clade I consists of TGA1 and TGA4; TGA2, TGA5, and TGA6 belong to clade II; and clade III contains TGA3 and TGA7 [11]. In *Arabidopsis*, only TGA1 and TGA4 interact with NPR1 in SA-induced leaves, while the rest TGAs constitutively interact with NPR1 [12]. Thus, all seven TGAs are the important components in the plant defence system.

Besides their involvements in plant defence, TGAs also act in plant development. For instance, when grown under low nitrate conditions, *tga1/tga4* shows altered root architecture [13, 14]. *TGA1* and *TGA4* are also found to be expressed around flower organ boundaries and required for inflorescence architecture, meristem maintenance and flowering [3].

In this study, we showed that TGA7 plays an important role in flowering time control. Loss of function of *TGA7* delayed flowering in *Arabidopsis*. In order to reveal the molecular mechanism of *TGA7* in flowering time control, the transcriptome changes between WT and *tga7* mutant seedlings at 9 DAG were analyzed by RNA-sEq. A total of 325 DEGs were identified, and four DEGs were associated with flowering time pathways. These results provide insights into the potential genes related to flowering time control in *tga7* mutant and will be useful for further study of molecular mechanisms of *TGA7* in floral transition.

Methods

Plant materials

Arabidopsis plants were grown on soil under long-day (LD; 16h/8h, light/dark) or short-day (SD; 8h/16h, light/dark) conditions at 23 °C. Mutants *gi-1*, *co-9*, *ft-10*, *svp-41*, Col:*FRI*^{SF2} (*FRI-Col*), *fld-3* and *fve-4* were all in the Col background [15, 16]. *fpa-7* (SALK_138449), *fca-2* (SALK_057540), *flk-1* (SALK_007750) and *tga7* (CS89835) seeds were bought from ABRC (the Arabidopsis Biological Resource Center, http://www.arabidopsis.org/).

Cleaved amplified polymorphic sequences (CAPS) analysis

A 689bp DNA fragment of *tga7* mutant or wild-type (WT) was amplified using primers as follows: Forward, 5'-TAAAGTTATCGCAGTTAGAGC-3'; Reverse, 5'-CCGCATCAATCACAATG-3'. PCR was carried out for 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute. Then the PCR products were digested by *Eco*RV and separated on 1% agarose-TAE gels.

Total RNA isolation

Isolation of total RNA was performed by using RNAprep Pure Plant Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. DNase I was added to the mixture to eliminate genomic and plastid DNA.

mRNA Library Construction

Total RNA was analyzed by using Nano Drop and Agilent 2100 bioanalyzer (Thermo Fisher Scientific, MA, USA). In order to purify mRNA, Oligo (dT) magnetic beads were used. Then, the mRNA was sheared into small fragments in the fragment buffer. The first-strand cDNA was synthesized by reverse transcription using random hexamer primers, then the second-strand cDNA by DNA polymerase. Afterwards, adapters were added to the double-stranded cDNA. In order to amplify the cDNA fragments, PCR was performed, and the resultant PCR products were purified and dissolved in Elution Buffer. After that, the PCR products

were heated denatured to get the final library. The sequencing was performed on BGIseq500 platform (BGI-Shenzhen, China). The transcriptome data sets have been submitted to the NCBI (accession number PRJNA649868).

De novo assembly and functional annotation of sequencing

The transcriptome data were filtered and analyzed according to published paper with minor modification [17]. Differential expression analysis and the significant levels of GO terms were all performed with Q value ≤ 0.05.

qRT-PCR

For expression analysis, 1 μg RNA was used for reverse transcription. The cDNA was synthesized by using FastKing gDNA Dispelling RT SuperMix kit (TIANGEN, Beijing, China) according to the manufacturers' instructions. qRT-PCR was performed by using UltraSYBR Mixture (with ROX; CWBio, Beijing, China) and the CFX96 real-time PCR detection system (Bio-Rad). Expression levels of detected genes were normalized to TUB2 expression. Error bars denote SD of three biological replicates [18]. The primers used for expression analysis are listed in Additional file 1.

Results

Regulation of flowering time by TGA7 in Arabidopsis

To reveal the function of *TGA7* in controlling flowering time, we analyzed the phenotype of *TGA7* using a *tga7* mutant that contain a point mutation in the seventh exon (Fig. 1a). The C to T mutation led to the loss of an *Eco*RV site in the *TGA7* gene, and resulted in an amino acid change from Ser to Leu in the TGA7 protein (Fig. 1a, b, Additional file 2). All *tga7* mutant plants delayed flowering compared to WT seedlings under both LD and SD conditions (Fig. 1c, d, e), suggesting that *TGA7* promoters flowering independently of the daylength conditions. We then also examined *TGA7* expression in different tissues of WT plants by qRT-PCR, and found that the highest expression of *TGA7* was in adult rosette leaves, and almost didn't expression in siliques (Fig. 1f).

Autonomous Pathway and thermosensory pathway Regulated TGA7 Expression

Since TGA7 is involved in floral transition, we then examined which flowering genetic pathways may relate to *TGA7* during flowering time control. The expression of *TGA7* remained steady in the photoperiod pathway mutants (Fig. 2a), and the phenotype of *tga7* mutant was delay flowering in LD and SD condition (Fig. 1c, d, e), suggesting that *TGA7* may not be involved in the photoperiod pathway. In addition, there were almost no effects on *TGA7* expression in gibberellin (GA) treatment (Fig. 2b). In both WT and *FRI-Col* plants, treatment of vernalization did not alter *TGA7* expression (Fig. 2c). These observations suggest that the GA and vernalization pathways also did not influence *TGA7*. By contrast, in

the autonomous pathway mutants, the *TGA7* expression was increased in *fca-2* and *fve-4*, decreased in *fld-3*and *flk-1* (Fig. 2d), suggesting that the autonomous pathway may affect *TGA7* expression.

SVP played crucial roles in the thermosensory pathway, *svp-41* mutant displayed steady flowering phenotype under different temperature conditions [19]. We then also analyzed *TGA7* expression in different temperature settings. *TGA7* expression increased with increasing temperatures (Fig. 2e). Furthermore, *TGA7* expression was steady in WT, *svp-41* and *35S:SVP* plants at 16℃, whilst *TGA7* expression was higher in *35S:SVP* but lower in *svp-41* at 23℃ (Fig. 2f). These findings demonstrate that thermosensory pathway may also regulate *TGA7* expression at ambient temperatures.

Transcriptomes of WT and tga7 mutant seedlings

To understand how *TGA7* affects flowering time, we identified downstream genes of *TGA7* that might be involved in its role in promoting flowering. To obtain a reference transcriptome for the WT and *tga7* mutant seedlings, three biological replicates were used for extraction of mRNA from WT and *tga7* mutant seedlings at 9 DAG, respectively. In total six RNA-seq libraries were constructed for transcriptome sequencing.

The raw data were qualified and filtered, yielding about 6.67 Gb of sequence data from each library (Additional file 3). By taking Pair-wise Pearson's correlation coefficients analysis, three replicates of each samples indicated that the sequencing data is highly repeatable (Fig. 3a). In order to gain an overview of the variations among these sequencing data, the principal components analysis (PCA) was performed, and the values of PC1 and PC2 were 97.58 and 2.21%, respectively (Fig. 3b). The PCA clearly separated the six RNA-seq libraries into two groups, WT and *tga7* mutant. The size distributions of mRNA are shown in Fig. 3c. The majority of mRNAs (84.55%) were between 500 bp and 3000 bp in length, only 1.60% of the mRNAs were > 5000 bp in length.

Identification of DEGs between WT and tga7 mutant seedlings

RPKM values were calculated to determine to the DEGs between WT and *tga7* mutant seedlings at 9 DAG. Totally, 325 DEGs were identified, among them, expression of 133 genes was induced and expression of 192 genes repressed (Fig. 4a). Among the 325 DEGs, AT3G55970, AT5G45570, AT5G44590, AT5G44440, AT4G12480 were the most up-regulated genes, while AT3G01345, AT4G36700, AT3G56980, AT5G28520, AT4G36700 were the most down-regulated genes. The heatmap in Fig. 4b showed the expression profiles of the DEGs between WT and *tga7* mutant seedlings. GO term enrichment analysis of these DEGs was performed and the top five largest GO terms in biological process were "photosynthesis, light harvesting in photosystem I", "photosynthesis, light harvesting", "protein-chromophore linkage", "photosynthesis" and "photosynthesis, light harvesting in photosystem II"; in molecular function, "chlorophyll binding", "protein domain specific binding", "RNA polymerase II regulatory region sequence-specific DNA binding", "hydrolase activity, acting on glycosyl bonds" and "carbohydrate kinase activity" were the five largest GO terms; and in cellular component, the top five largest GO terms were "photosystem I", "photosystem II", "plastoglobule", "chloroplast thylakoid membrane" and "chloroplast" (Fig. 4c).

Identification Of Key Flowering Time-related Degs

A large number of genes are flowering time-related, and play vital roles in floral transition, an important turning point from vegetative growth to reproductive growth [20–22]. Among 325 DEGs which were identified between WT and *tga7* mutant seedlings (Fig. 4), 4 DEGs were involved in flowering time pathways. The expression level of *FLC*, *MAF5* and *SMZ* were up-regulated, while *NF-YC2* was down-regulated in *tga7* mutant seedlings, compare to WT seedlings (Additional file 4).

Validation Of The Expression Of Flowering Time-related Degs

To validate the expression of the 4 flowering time-related DEGs (*FLC*, *MAF5*, *SMZ* and *NF-YC2*) identified by RNA-seq (Additional file 4), three independent biological duplicates of WT and *tga7* mutant seedlings collected at 9 DAG were analyzed by qRT-PCR assay. The expression levels and tendency of the four flowering-related DEGs were consistent with RNA-seq results (Fig. 5). This result suggests that the data gained by RNA-seq are reliable.

Discussion

In the present study, it was found that loss of function of *TGA7* showed delay-flowering phenotype in *Arabidopsis* (Fig. 1). To uncover the role of *TGA7* in flowering time control, transcriptomic analyses between WT and *tga7* mutant seedlings at the same developmental stage (9 DAG) revealed 325 DEGs, of which *NF-YC2, SMZ, MAF5* and *FLC* were involved in flowering time pathways (Fig. 5; Additional file 4).

NF-Y, a heterotrimeric TF family, consists of three subfamilies, NF-YA, NF-YB and NF-YC. NF-YB and NF-YC form dimer with a histone fold domain, whilst NF-YA confers to sequence specificity [23, 24]. The heterotrimeric NF-Y complex binds to promoters with *CCAAT* elements, and then regulates expression of the target genes [23, 24]. In yeast and mammals, although each member of the NF-Y family is encoded by a single gene, they can be spliced to multiple isoform, followed with post-translational modifications [25, 26]. In mammals, the NF-Y complex plays important roles in many processes including endoplasmic reticulum stress, DNA damage, and cell cycle regulation [27–29]. However, in plants every NF-Y is encoded by multiple genes, and then forms sub-families [30]. There are 10 *NF-YA*, 13 *NF-YB*, and 13 *NF-YC* in *Arabidopsis* genome [31]. Similar to other plant TFs, duplicate members in the NF-Y family also displayed similar functions in *Arabidopsis* [30, 32]. In the past decades, it has been showed that the NF-Y complex plays crucial roles in plant stress responses, growth and development [26, 30, 33].

NF-Y genes including *NF-YB2*, *NF-YB3*, *NF-YC3*, *NF-YC4* and *NF-YC9* were involved in photoperiod pathway in *Arabidopsis*, [34–37]. Single *nf-y* mutant did not show any obvious flowering phenotype, whilst double or triple mutants such as *nf-yb2-1 nf-yb3-1* or *nf-yc3-2 nf-yc4-1 nf-yc9-1* delayed flowering [37]. Considering that *NF-YC2* is in the same subfamily as *NF-YC3*, *NF-YC4*, and *NF-YC9*, it would be

expected they may possess similar functions in photoperiod-dependent control of flowering-time. However, *tga7* exhibited a delay-flowering phenotype under both LD and SD conditions (Fig. 1c, d, e), suggesting that later flowering in *tga7* was independent of photoperiod pathway. Thus, down-expression of *NF-YC2* may not the main reason for delay-flowering seen in the *tga7* mutant plants.

SMZ (*SCHLAFMÜTZE*), together with its paralog *SNZ* (*SCHNARCHZAPFEN*), belongs to the AP2-type transcription factor family that repress flowering. Both *SMZ* and *SNZ* are the target of *miR172*, an important regulator in the ageing pathway [38]. *SMZ* delays flowering under LD condition. When expressed in the leaves, SMZ is able to repress flowering by directly binding to the *FT* genomic locus, down-regulating *FT* expression [38, 39]. Thus, the elevated expression level of *SMZ* may at least partially account for the delayed flowering in the *tga7* mutant.

Furthermore, expression levels of *FLC* and *MAF5* were all increased in *tga7* mutant. *FLC*, encoding an MADS-box protein, is one of the most critical repressors in the flowering regulatory network [40–42]. MAF1-5 are five FLC homologs in *Arabidopsis*, FLC and MAF1-5 all belong to MADS-box transcription factor, and repress floral transition [43]. Many flowering regulatory genes in autonomous pathway promote flowering by directly repressing *FLC* expression, and the mutants of these genes including *FLD* and *FLK* in autonomous pathway showed delay-flowering phenotype under both LD and SD conditions [44–46].

FLD encodes a histone demethylase, a homolog of the human LSD1 (histone H3K4 demethylase) in *Arabidopsis* [47, 48]. It represses *FLC* expression via histone modification [47–50]. *FLD* can physically interact with *FPA* and *FCA*, two autonomous pathway genes [50]. The roles of *FCA* and *FPA* on regulating *FLC* expression and floral transition may depend on *FLD* [50, 51]. Moreover, *FLD* also interacts with HDA5 and HDA6, two histone deacetylases, to regulate *FLC* expression. *FLK* contains RNA-binding domains and only exists in plants [52, 53]. FLK may repress *FLC* expression level by binding *FLC* RNAs [54, 55]. However, how FLD and FLK regulate *FLC* expression needs further investigation. Here, we found that the *TGA7* expression was decreased in *fld-3* and *flk-1* (Fig. 2d). Considering that expression levels of *FLC* and *MAF5*, the closest homolog of *FLC*, increased dramatically in *tga7* compared with WT seedlings (Additional file 4, Fig. 5), and that *tga7* mutant displayed a delay-flowering phenotype under both LD and SD conditions (Fig. 1c, d, e), we propose that *FLD* and *FLK* may regulate *FLC* expression through *TGA7*.

Conclusions

In summary, 6 cDNA libraries from WT and *tga7* mutant seedlings at 9 DAG were constructed for sequencing independently. Through bioimformatics mining, 325 DEGs were identified; of which four genes *NF-YC2, SMZ, MAF5* and *FLC* were associated with flowering time control. Differential expression of these flowering time-related genes were analyzed and validated by qRT-PCR. Among them, *FLC* and *MAF5* may be mainly responsible for the delay-flowering phenotype in *tga7*, as *TGA7* expression was regulated by autonomous pathway genes. We envisage that further studies will elucidate how *TGA7*

impacts on *FLD* and *FLK* to regulate *FLC* expression and deepen our knowledge into the autonomous pathway in control of flowering.

Abbreviations

CAPS: Cleaved amplified polymorphic sequences; DAG: days after germination; DEGs: differentially expressed genes; GA: gibberellin; GO: gene ontology; LD: long-day; NPR1: non-repressor of pathogenesis-related gene 1; PCA: principal components analysis; qRT-PCR: quantitative real-time PCR; RNA-seq: RNA sequencing; RPKM: reads per kb per million reads; SD: short-day; SMZ: SCHLAFMÜTZE; SNZ: SCHNARCHZAPFEN; TGA: TGACG-Binding transcription factor; WT: wild-type;

Declarations

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Availability of data and materials

The datasets supporting the conclusions of this article are available in the NCBI Short Read Archive with accession number PRJNA649868.

Authors' contributions

CQ and NNS designed the experiments. XRX, JYX, CY, QQC and PCZ performed the experiments, XRX and CY carried out the qRT-PCR analysis. CQ, YKH and QGL analyzed the data. CQ drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

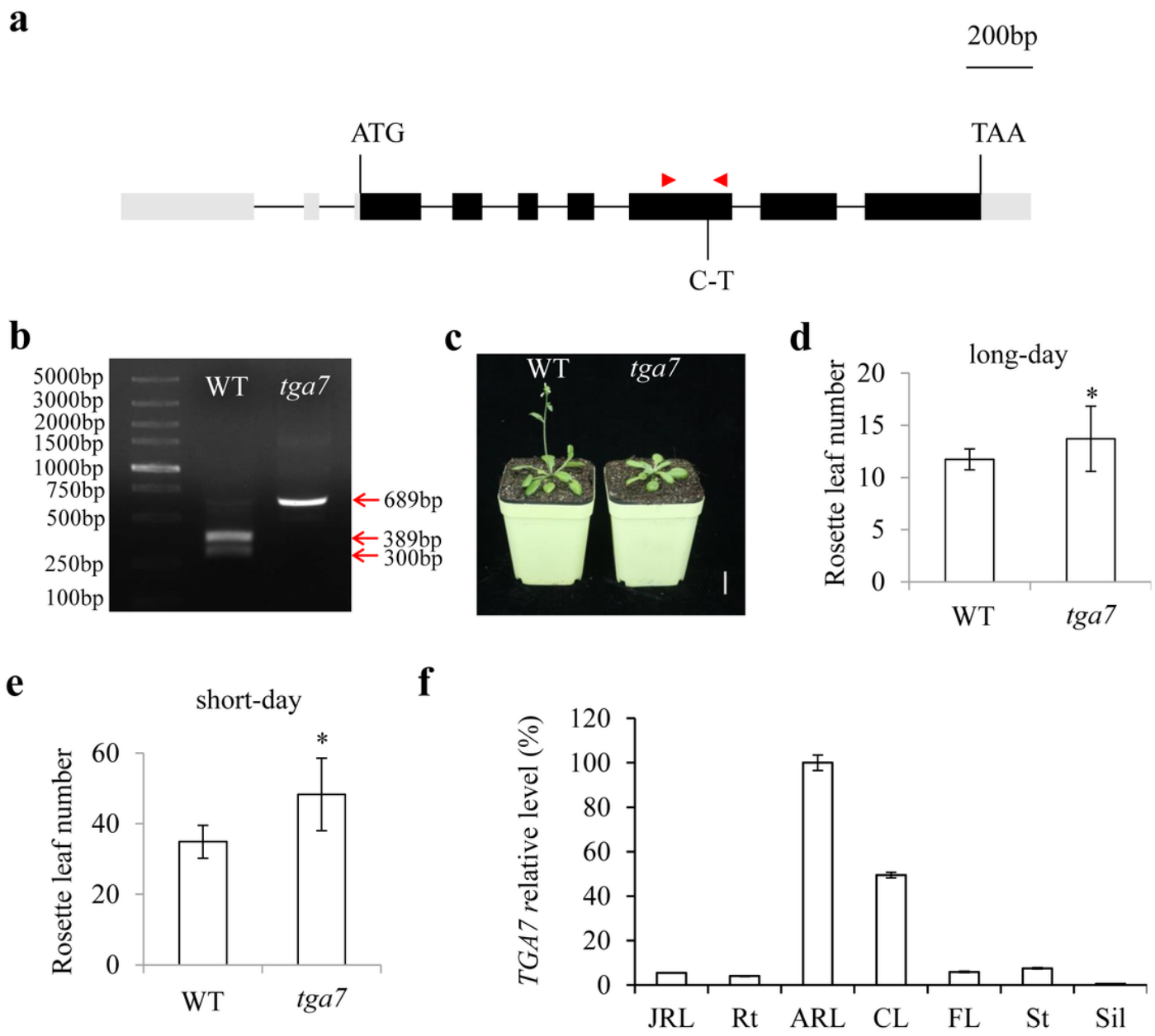

Figure 1

TGA7 regulates flowering time in Arabidopsis. a The structure of TGA7 coding region. Black boxes represent exons, gray boxes represent untranslated regions, and black lines indicate introns. The point mutation is listed underneath. Red arrowheads indicate the positions of primers used in Fig. 1b. b The cropped gels of CAPS analysis of wild-type and tga7 mutant. Genomic DNA of wild-type and tga7 mutant was amplified using CAPS marker list in Additional file 1, and then the PCR products were digested with EcoRV. c tga7 shows delay-flowering phenotype under long-day conditions. Scale bar: 2 cm. d and e Flowering time of tga7 grown under long-day (d) and short-day conditions (e). Values were represented from at least 10 plants showing specific genotypes. Asterisks indicate significant differences between WT and tga7 mutant in flowering time (Student's t test, $p \leq 0.05$). f The expression level of TGA7 in

various tissues of WT plants is analysis by qRT-PCR analysis (n=3, ±SD). JRL, juvenile rosette leaves; Rt, roots; ARL, adult rosette leaves; CL, cauline leaves; FL, flowers; St, inflorescence stems; Sil, siliques.

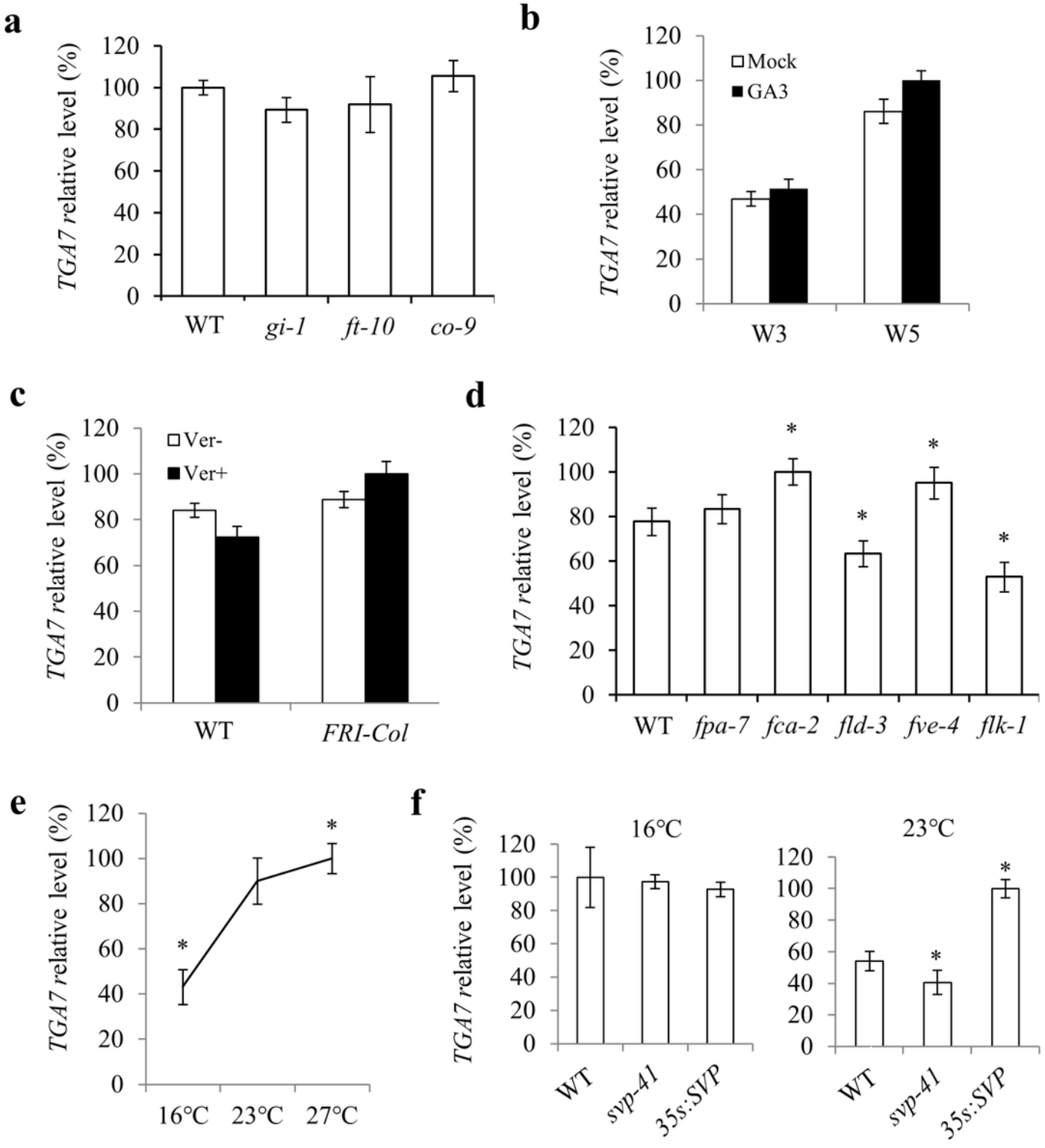

Figure 2

TGA7 expression is regulated by several pathways. a TGA7 expression in photoperiod-pathway mutants at 9 DAG. b TGA7 expression under GA treatment. WT seedlings grew under short-day conditions for 2 weeks, then these seedlings were treated with 100 μM GA3 or 0.1% ethanol weekly, then seedlings treated

for 3 weeks (W3) and 5 weeks (W5) were collected for further analysis. c TGA7 expression with vernalization treatment. The seedlings are vernalized at 4°C for 8 weeks. 9-day-old seedlings were collected for further analysis. d TGA7 expression in autonomous-pathway mutants at 9 DAG. e TGA7 expression level in WT seedlings grown at 16°C, 23°C, and 27°C under LD conditions at 9 DAG. f TGA7 expression in WT, svp-41, and 35S:SVP grown at 16°C and 23°C under LD conditions at 9 DAG. Asterisks indicate significant differences (Student's t test, $p \leq 0.05$).

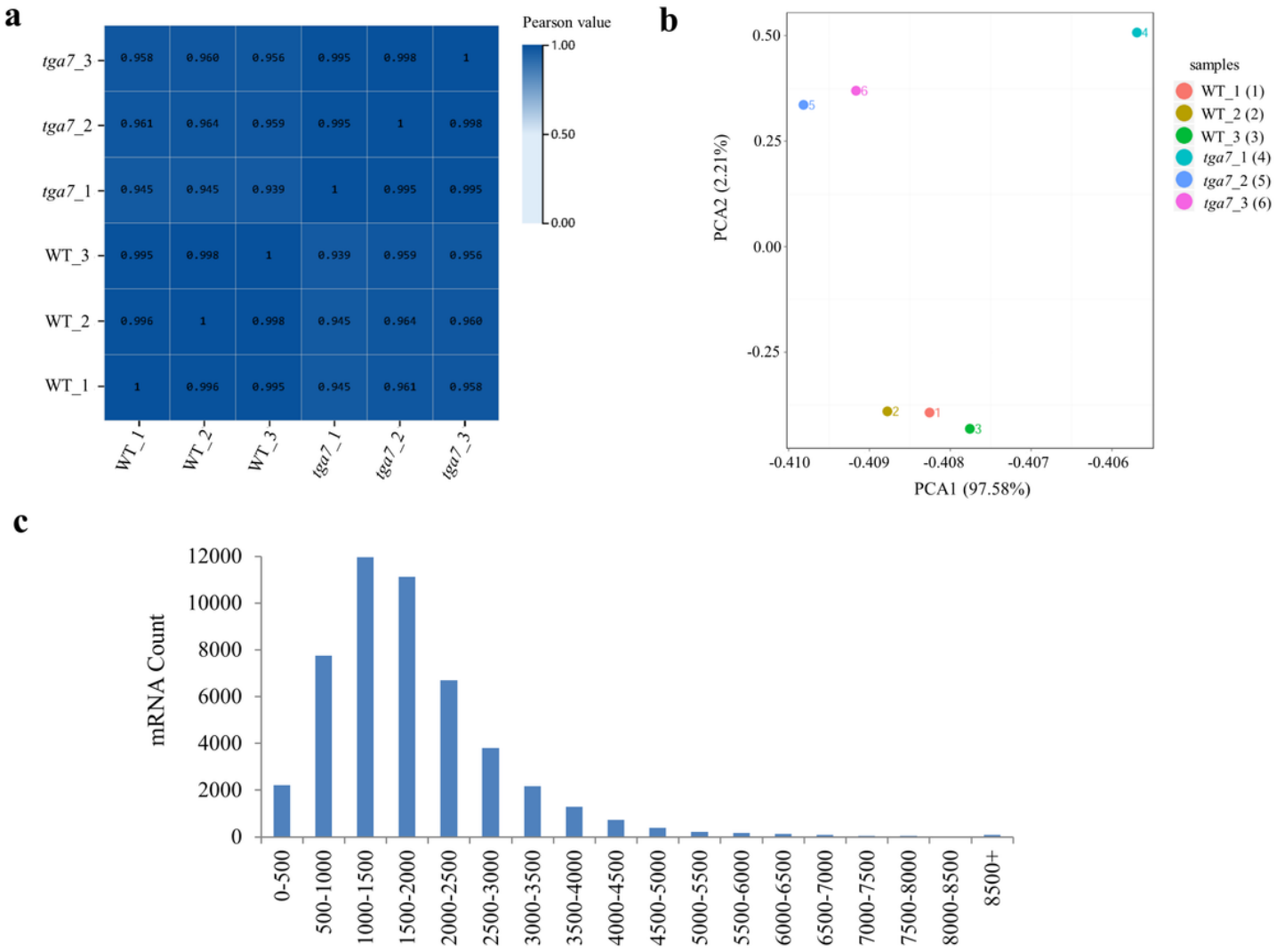

Figure 3

Transcriptomes of WT and tga7 mutant seedlings. a Pair-wise Pearson's correlation coefficients analysis shows that the sequencing data from three replicates X two samples is highly repeatable. b Principal components analysis of the transcriptomes. c The size distributions of mRNA of the transcriptomes.

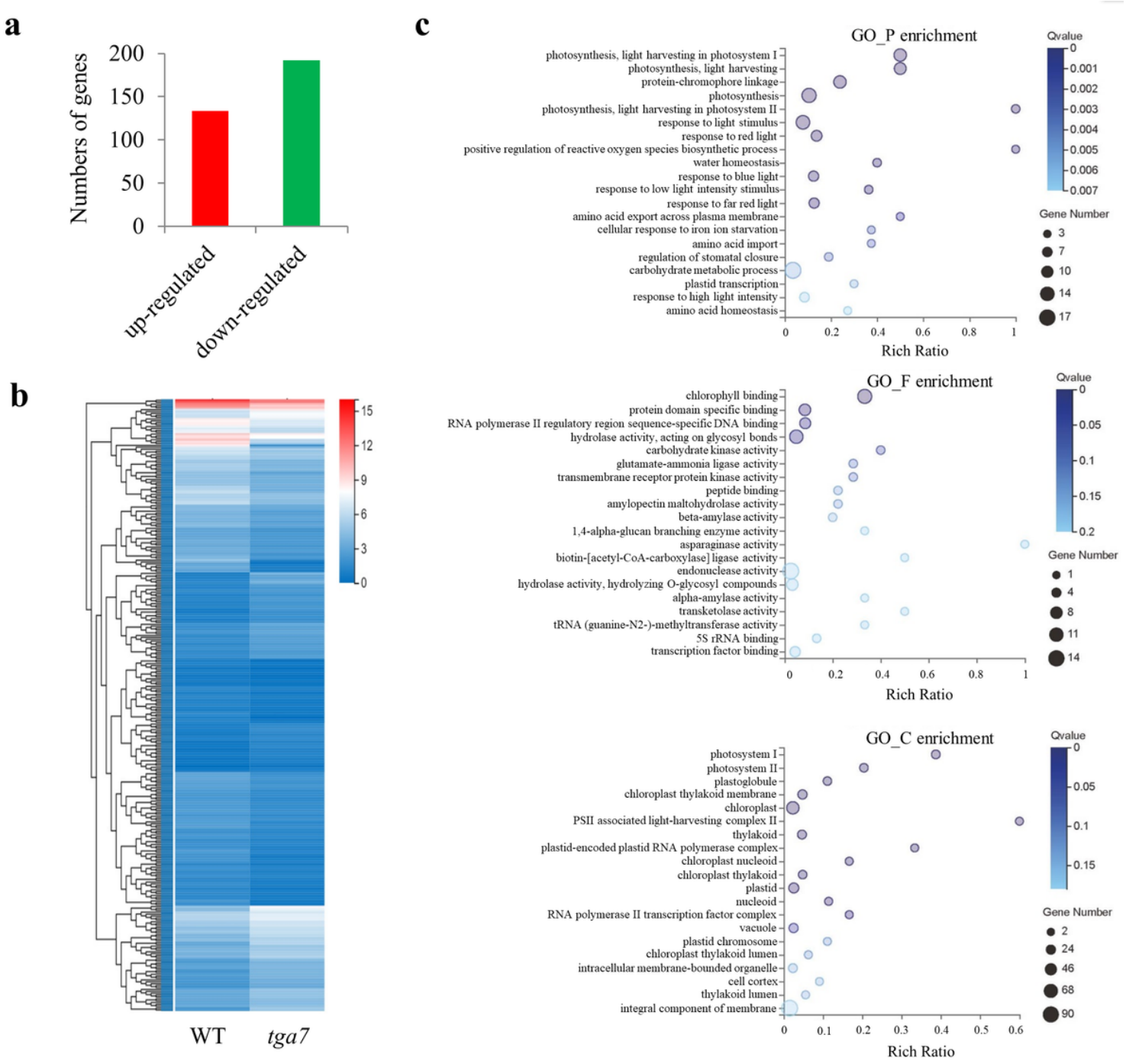

Figure 4

Transcriptional profiles in WT and tga7 mutant seedlings at 9 DAG. a The numbers of genes that were up-regulated or down-regulated between WT and tga7 mutant seedlings at 9 DAG. b Expression profiles of the DEGs between WT and tga7 mutant seedlings at 9 DAG were showed by a heatmap. c GO term enrichment analysis of the DEGs between WT and tga7 mutant seedlings.

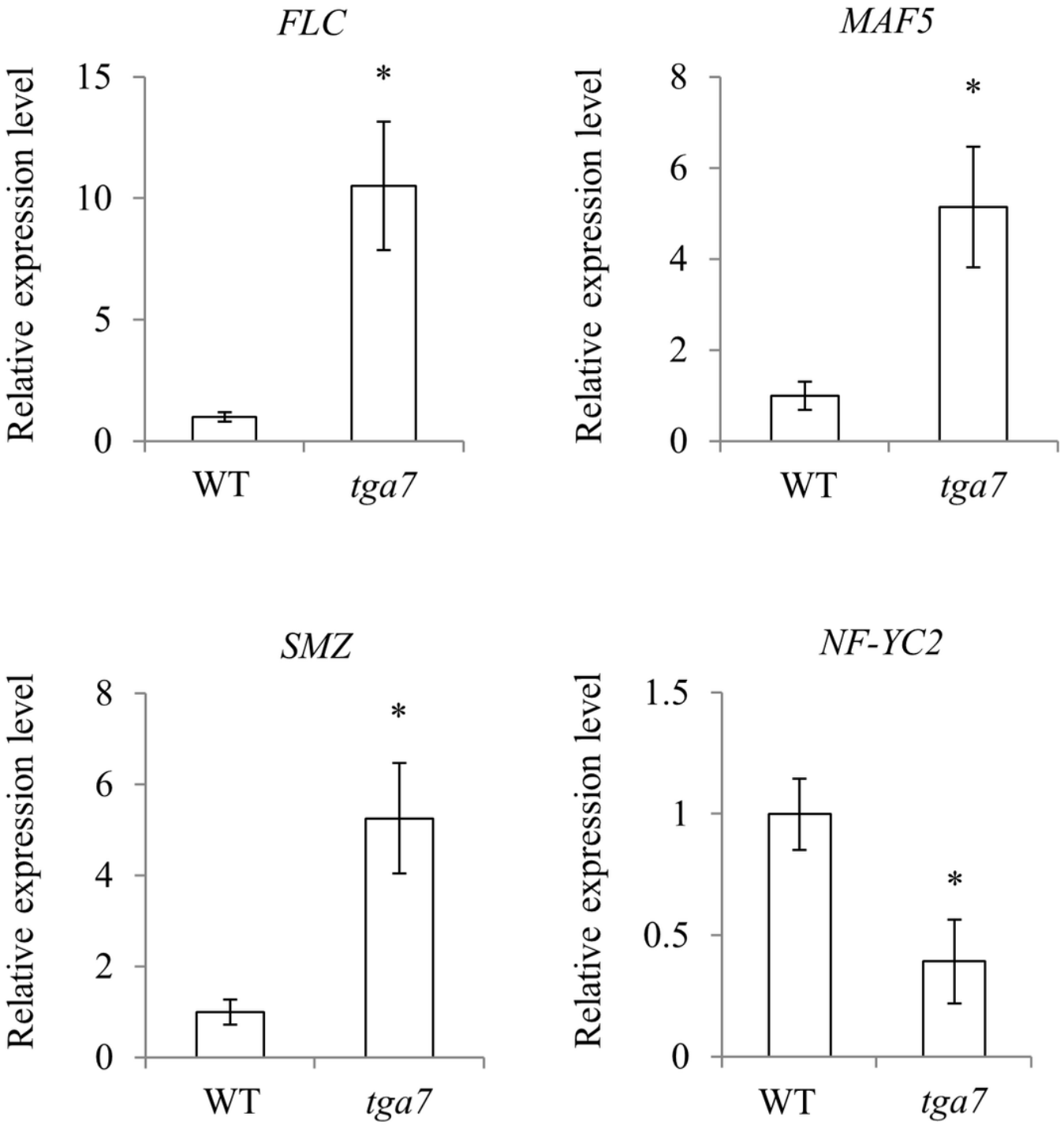

Figure 5

Quantitative real-time PCR validation of flowering time-related DEGs. Expression levels in all panels determined by qRT-PCR and then normalized to TUB2 expression. The data were analyzed by three independent repeats. Error bars denote SD. Asterisks indicate significant differences (Student's t test, $p \leq 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile5.docx
- Additionalfile4.xlsx
- Additionalfile3.xlsx
- Additionalfile2.tif
- Additionalfile1.xlsx