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ORIGINAL ARTICLE

Silencing *SlAGL6*, a tomato *AGAMOUS-LIKE6* lineage gene, generates fused sepal and green petal

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Abstract

Key message **Silencing *SlAGL6* in tomato leads to fused sepal and green petal by influencing the expression of A-, B-class genes.**

Abstract *AGAMOUS-LIKE6* (*AGL6*) lineage is an important clade MADS-box transcription factor and plays essential roles in various developmental programs especially in flower meristem and floral organ development. Here, we isolated a tomato *AGL6* lineage gene *SlAGL6* and successfully obtained several RNA interference (RNAi) lines. Silencing *SlAGL6* led to abnormal fused sepals and light green petals with smaller size. The total chlorophyll content in transgenic petals increased and the morphology of epidermis cells altered. Further analysis showed that A-class gene *MACROCALYX* (*MC*) participating in sepal development and a NAC-domain gene *GOBLET* involving in boundary establishment were down-regulated in transgenic lines. In transgenic petals, two chlorophyll synthesis genes, *Golden2-like1* (*SlGLK1*) and *Golden2-like2* (*SlGLK2*), two photosystem-related genes, *ribulose bisphosphate carboxylase small chain 3B* (*SlrbcS3B*) and *chlorophyll a/b-binding protein* 7 (*SlCab-7*) were induced and three B-class genes *TM6, TAP3* and *SlGLO1* were repressed. These results suggest that *SlAGL6* involves in tomato sepal and petal development.

Keywords MADS-box transcription factor · *AGAMOUS-LIKE 6* · Floral organ · Tomato

Introduction

Decades ago, ABC model was proposed to illustrate the relationship between genes and the formation of floral organs according to the research on various flower mutations of model plants. In ABC model, genes in three classes control the initiation of four floral organs: sepals, petals, stamens and carpels and the development of four

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whorls are separately determined by A class alone, A and B together, B and C together and C alone (Coen and Meyerowitz 1991; Schwarzsommer et al. 1990). In *Arabidopsis*, the A-class contains two genes *APETALA1* (*AP1*) and *APETALA2* (*AP2*), B-class contains *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) and C-class contains *AGAMOUS* (*AG*). A few years later, the model was expanded by finding out D- and E-class genes in the process of flower development. D-class is involved in ovule development (Angenent et al. 1995; Colombo et al. 1995), while E-class genes are considered participating in the identity of flower meristem and all flower organs (Angenent et al. 1994; Ditta et al. 2004; Pelaz et al. 2000; Vandenbussche et al. 2003). E function is controlled by *SEPALLATA* (*SEP*) clade genes, previously known as *AGAMOUS LIKE 2* (*AGL2*) clade (Pelaz et al. 2001; Theissen 2001). All these genes belong to MADS-box family except for *AP2* and its homologs (Theissen et al. 2000). Further study revealed that besides *SEP* clade, MADS-box gene *AGL6* and its homologs also function like *SEP* genes (Hsu et al. 2014; Rijpkema et al. 2009; Smaczniak et al. 2012).

AGL6 clade genes have been proved functioning in manifold developmental events, especially in controlling flower development. For example, *AGL6* lineage members have been confirmed to play role in lateral organ initiation (Koo et al. 2010), flowering time regulation (Koo et al. 2010; Yoo et al. 2011b), circadian-clock control (Yoo et al. 2011a) and male and female gametophyte morphogenesis construction (Hsu et al. 2014) in *Arabidopsis*. Ectopic expression of wintersweet and orchid *AGL6* gene in *Arabidopsis* also alter flowering time and morphology of floral organs (Hsu et al. 2003; Wang et al. 2011). Rice *AGL6* lineage gene *MADS6* has been proved interacting with multiple floral homeotic genes to specifying floral organ identities and meristem fate (Duan et al. 2012, Hsu et al. 2014; Li et al. 2011, 2010). Maize *AGL6* mutation *bearded-ear* (*bed*) exhibited extra mosaic or fused floral organs in upper floral meristem and additional floral meristems in lower floral meristem (Thompson et al. 2009). In *Petunia*, research on mutants clearly demonstrated that *PhAGL6* (*pMADS4*) functions redundantly in petal and anther development with E-function genes *FBP2* and *FBP5*, in addition, *PhAGL6* plays role in ovule development (Rijpkema et al. 2009). In tomato, recent research revealed that *Slagl6* mutations confer facultative parthenocarpic, leading to high quality seedless fruits under heat stress conditions (Klap et al. 2016), which indicates that *SlAGL6* plays an essential role in the regulation of fruit set.

According to phylogenetic analysis, *AGL6* is sister to *SEP* and *SQUAMOSA* (*SQUA*, also known as *AP1*) (Hileman et al. 2006; Kim et al. 2013). *AGL6* lineage is considered as an ancient clade. No distinctive *AGL6* members have been found in ferns so far and *AGL6* exists in both gymnosperms and angiosperms indicating that the emergence of *AGL6* lineage dates back to the initiation of seed plants about 400 MYA (million years ago) (Theissen et al. 2000). Along the evolutionary process of *AGL6* lineage, several duplications were proposed and four duplications were proved: one at the base of core eudicots, one during basal angiosperm diversification and two in monocot evolution which suggest that the function of *AGL6* become diversified (Viaene et al. 2010). Duplication usually causes subfunctionalization and new function. It is considered as a strong positive selection pressure on *AGL6* by functioning redundantly with *SEP* but exiting stably in different angiosperm lineages which also indicating that function of *AGL6* is not exactly identical to *SEP* genes (Dreni and Zhang 2016).

Though the protein sequence of AGL6 is conserved among angiosperms, the functions are not the same in different species. To know more comprehensively about the function of *AGL6* lineage genes in floral organ development, the study on diverse species is necessary. Previous study showed tomato *Slagl6* mutations have effect on parthenocarpic fruits in tomato cv. M82 (Klap et al. 2016), but precise functional study of *SlAGL6* on floral organ and inflorescence has not been reported. To explore the function of *SlAGL6* in flower development, we used tomato (*Solanum lycopersicon*) Mill. cv. Ailsa Craig^{++}, a common material for tomato research, as material to detect the expression of *SlAGL6* in different tissues, floral organs and flowers at different developmental stages. Transgenic tomato plants with *SlAGL6* silenced were also obtained by RNA interference using *S. lycopersicon* Mill. cv. Ailsa Craig^{++} as wild-type tomato. Down-regulation of *SlAGL6* produced fused sepals and pale green petals indicating that *SlAGL6* plays an important role on the development of floral organs.

Materials and methods

Plant material and growth condition

Tomato plants (*S. lycopersicon* Mill.cv. Ailsa Craig^{++}) were grown in controlled greenhouse conditions of 16-h-day (25 °C)/8-h-night (18 °C). Materials of roots, stems, young leaves, mature leaves, senescent leaves, flowers and fruits of different stages were collected from tomato plants. Flower parts (sepals, petals, stamens and carpels) were collected from flowers at anthesis. Different stages of flowers were determined by size (2, 5, 7, and 10 mm) before anthesis and by time after anthesis. Samples were frozen in liquid nitrogen immediately and stored at −80 °C for expression analysis.

Total RNA extraction and quantitative real-time PCR analysis

Total RNA was isolated using Trizol (Takara, Dalian, China) according to the manufacture's instructions. M-MLV reverse transcriptase (Promega, Beijing, China) was used for the synthesis of first strand cDNA with oligo $(dT)_{18}$ as primer. Quantitative RT-PCR was performed using CFX96™ Real-Time System (Bio-Rad, USA). The 10 μL reaction system contained 5 μL 2× SYBR Premix Ex Taq (Takara), 0.5 μL primers, 1 μL cDNA and 3.5 μL of distilled deionized water. Tomato *CAC* gene was selected as internal standard for tissue-specific expression analysis (Exposito-Rodriguez et al. 2008). All primers used in this study are exhibited in Supplementary Table S1. The analysis of relative gene expression levels were performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Samples of three biological repeats were employed in this study.

Structure and phylogenetic analysis of *SlAGL6*

Multiple sequence alignment of SlAGL6 with other MADS-box proteins was conducted by DNAMAN programs. Phylogenetic tree of SlAGL6 and other reported proteins was constructed using MEGA5.05 software. The accession numbers of proteins contained in multiple sequence alignment and phylogenetic analysis are listed in Supplementary Table S2.

Construction of *SlAGL6* RNAi vector and plant transformation

To repress the expression of *SlAGL6* gene, we constructed an RNAi vector. Primers of *SlAGL6*-i-F and *SlAGL6*-i-R (sequences are listed in Supplementary Table S1) were used for the amplification of specific DNA fragment of *SlAGL6* gene. The purified products of 393 bp were digested with *Xho* I/ *Kpn* I and inserted into pHANNIBAL plasmid at *Xho* I/*Kpn* I restriction site in the sense orientation while products tailed with *Xba* I/*Cla* I restriction enzyme in the antisense orientation. At last, the double-stranded RNA expression unit, containing the cauliflower mosaic virus (CaMV) 35 S promoter, *SlAGL6* fragment in the sense orientation, PDK intron, *SlAGL6* fragment in the antisense orientation and OCS terminator was digested by *Sac* I/*Xba* I and linked into plant binary vector pBIN19 in *Sac* I/*Xba* I restriction sites.

The generated binary plasmids were translated into *Agrobacterium tumefaciens* LBA4404 strain, and *A. tumefaciens*-mediated transformation procedure according to the methods of Chen et al. (2004) was applied to WT tomato cotyledon explants. The transgenic plants were screened by kanamycin (50 mg L^{-1}) resistance and detected with primers *NPTII*-F and *NPTII*-R (sequences are listed in Supplementary Table S1). The positive transgenic lines were screened out and prepared for the subsequent experiments.

Extraction and quantitation of chlorophyll in petals

Petals of WT and transgenic lines were milled into powder with liquid nitrogen after weighting. Samples were transferred into tubes and 5 mL 80% of acetone was added for extracting. The extraction was centrifuged at 5000 rpm for 15 min at 4 °C after 24 h in dark. The supernatant was carefully moved out and the absorbance at 645 and 663 nm was recorded. Total chlorophyll was calculated using formulas: Chl (mg g^{-1}) = (20.29 × A645 + 8.02 × A663) (Wellburn 1994).

Statistics of petal length and fused sepals

Petal length of WT and transgenic flowers were measured at 2 dpa (day post anthesis) and the flowers employed in statistics were all the first flowers of inflorescences. The fused sepals were observed at 2 dpa. At least ten flowers per lines were contained in statistics.

Anatomical analyses of flowers

Flowers at 2 dpa were fixed by 70% ethanol/acetic acid/formaldehyde (18:1:1, by volume; FAA). The samples were conducted with dehydration, paraffin embedding, sectioning and dewaxing in proper order. The cross section of one third from bottom of flowers was observed by microscope (OLYMPUS IX71) and photographed.

Scanning electron microscopy

Flowers of wild type and transgenic plants at anthesis were fixed in FAA and then dehydrated in gradient ethanol–water series. After vacuum drying, petals were separated and sputtered gold for scanning electron microscopy observation and photography.

Statistical analyses

Data were presented as mean ± standard deviation. Significant difference between transgenic lines and WT was analyzed using Student's *t* test (*$P < 0.05$).

Results

Sequence and expression analysis of *SlAGL6* gene

To investigate the function of tomato *AGL6* lineage in floral organ development, we concentrated our work on *SlAGL6* (GenBank accession number: XM_004229820.1). Sequence analysis manifested that *SlAGL6* contains an open reading frame (ORF) of 759 bp encoding 253 amino acids. Multiple sequence alignment with other typical MADS-box proteins indicated that SlAGL6 is a conservative MADS-box protein (Fig. 1a) sharing 85.2% identity with *Petunia* PhAGL6. Phylogenic analysis implied that SlAGL6 belongs to conserved AGL6 lineage which is a sister clade of SEP class (Fig. 1b).

In addition, the expression profile of *SlAGL6* was investigated in various tomato tissues (including roots, stems, young leaves, mature leaves, senescent leaves, flowers and fruits in different stages) and four floral organs (sepals, petals, stamens and carpels). Interestingly, we found that the expression levels of *SlAGL6* in flowers were significantly higher than in other tissues (Fig. 1c). Furthermore, the profile of *SlAGL6* in four floral organs revealed the highest transcript level appeared in petals and carpels, followed by sepals, and the lowest in stamens (Fig. 1d). These results indicated the possibility of *SlAGL6* participating in the development of petals, carpels and probably in sepals. The expression of *SlAGL6* existed in the whole development process of flowers and the expression at and post anthesis was higher than pre-anthesis. The transcript accumulation of *SlAGL6* reached peak at anthesis (Fig. 1e).

Repression of *SlAGL6* produces fused sepals

Tomato transgenic plants expressing the sense and antisense RNA of *SlAGL6* were generated to examine the

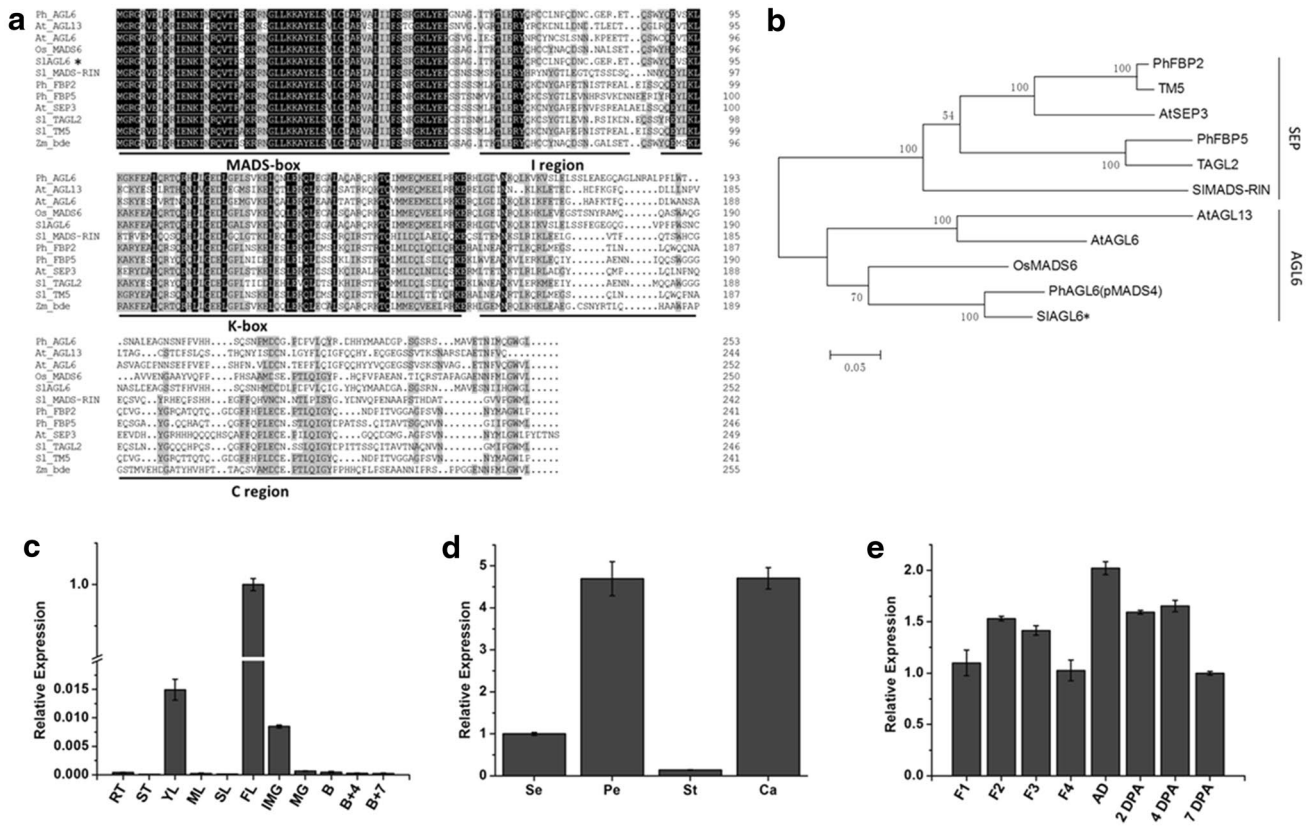

Fig. 1 Sequence, phylogenic and expression analysis of SlAGL6 gene. **a** Multiple sequence alignment of SlAGL6 with other MADS-proteins. SlAGL6 is marked with *asterisk*. **b** Phylogenic analysis of SlAGL6 and other MADS-box proteins. The phylogenic tree was constructed by the neighbor-joining method, bootstrap analysis of 1000 replicates. SlAGL6 is marked with *asterisk*. Accession numbers of other proteins contained in phylogenic analysis are listed in Supplement Table S2. **c** Expression profile of *SlAGL6* in WT. *RT* root, *ST* stem, *YL* young leaf, *ML* mature leaf, *SL* senescent leaf, *FL* flower, *IMG* immature green fruit, *MG* mature green fruit, *B* breaker fruit, *B + 4* 4 days after breaker, *B + 7* 7 days after breaker. **d** Expression profile of SlAGL6 in four-whorl floral organs of WT. *Se* sepal, *Pe* petal, *St* stamen, *Ca* carpel. Four floral organs were sampled at 2 dpa. **e** Expression of *SlAGL6* in different stages of flower development. *F1* 2 mm flower, *F2* 5 mm flower, *F3* 7 mm flower, *F4* 10 mm flower, *AD* anthesis day, *2 dpa* 2 days post anthesis, *4 dpa* 4 days post anthesis, *7 dpa* 7 days post anthesis. Each value represents the mean ± SE of three replicates

function of *SlAGL6* and seven transgenic lines were obtained. To further determine the repression efficiency of *SlAGL6* in transgenic lines, total RNA of flower was extracted and qRT-PCR was conducted. The result displayed that *SlAGL6* in three transgenic lines (RNAi2, RNAi6 and RNAi11) was down-regulated by more than 80% (Supplementary Fig. 1) compared with the control and the three transgenic lines were selected for subsequent experiments. In each floral organ, *SlAGL6* was down-regulated by more than 80% while in sepals, the efficiency of silencing reached 90% (Supplementary Fig. 1).

In *SlAGL6* silenced lines, the sepals of almost all flowers fused in varying degrees (Fig. 2c, d) while the normal sepals split completely each other (Fig. 2b). Some sepals fused together all along, even when the flower completely opened. Petals could not unfold normally due to the constraint of fused sepals. The fused sepals in *SlAGL6*-RNAi lines were easily separated by hand. By paraffin section, the cross section showed that sepals of transgenic lines at 2 dpa jointed each other by few layers of cells (Fig. 2f) while WT sepals was separated (Fig. 2e). A quantitative statistics of fused sepals was conducted. We defined two sepals adhering less than one third of sepal length as normal dehiscence while more than one third as fusion. The data illustrated that the numbers of sepals in WT and transgenic line had no difference, but the numbers of normal dehisced sepals in transgenic lines were obviously inferior to WT (Supplementary Table S3).

Transcription of genes concerned with sepal development are repressed

To figure out the possible genes affected in sepals of transgenic lines, we examined the transcription of a known A-class gene *MC* (Vrebalov et al. 2002) as well as two reported genes *GOBLET* (Berger et al. 2009) and *SlNAM2* (Hendelman et al. 2013) that can also lead to sepal fusion in sepals of 2 dpa. For *MC*, the expression reduced by half in transgenic lines compared with WT (Fig. 3a). The transcription of *GOBLET* was slightly down-regulated in transgenic lines (Fig. 3b) while *SlNAM2* had no apparent difference in transcriptional

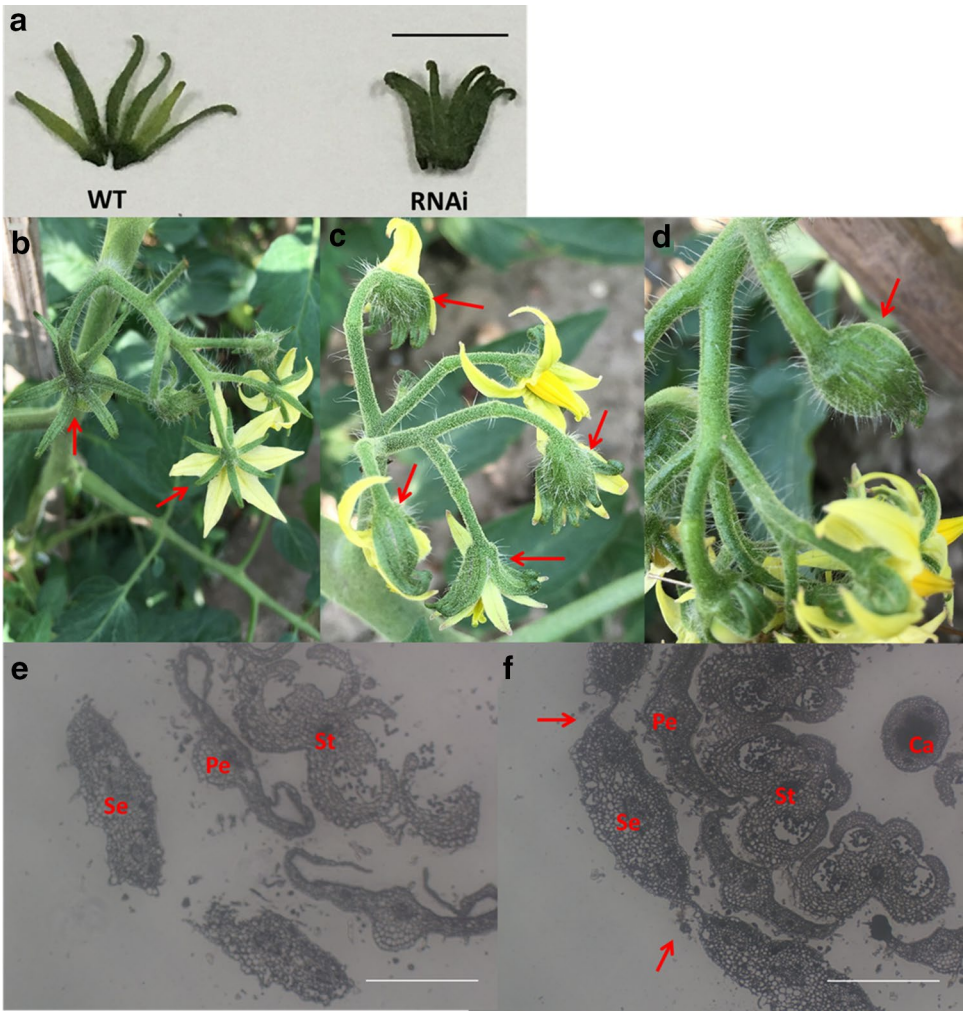

Fig. 2 Phenotype of sepal in SlAGL6 RNAi lines. **a** Sepals of transgenic lines are fused (separated from flower) *Bars* 1 cm. **b–d** Normal sepals of WT (**b**) and fused sepals (**c, d**) in transgenic lines. **e, f** Cross sections of WT (**e**) and transgenic (**f**) flowers at 2 dpa by paraffin section. *Arrows mark* the connectives between sepals. *Se* sepal, *Pe* petal, *St* stamen, *Ca* carpel. *Bars* 9 μm

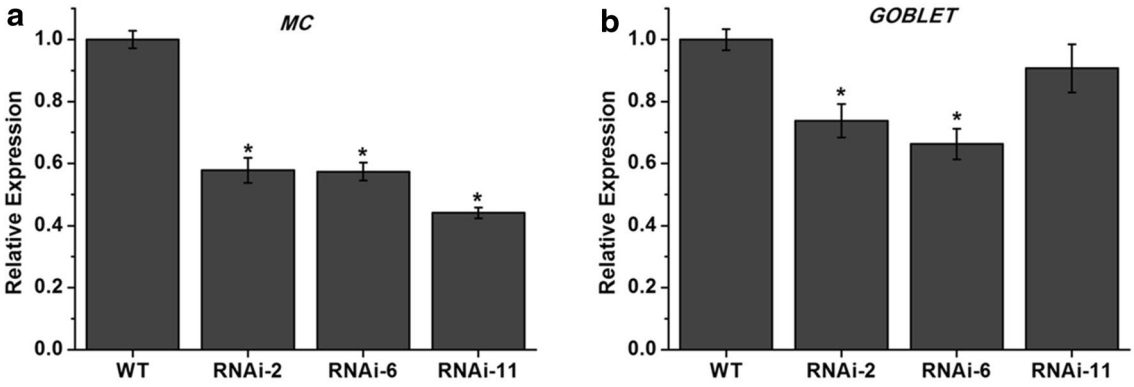

Fig. 3 Expression analysis of genes involving in sepal development in WT and transgenic lines. Relative expression of *MC* (**a**) and *GOBLET* (**b**) in sepal, sepals were obtained at 2 dpa. Each value represents the mean ± SE of three replicates. *Asterisks* indicate a significant difference ($P < 0.05$) between WT and transgenic lines

level (Supplementary Fig. S2a). Together, repression of *SlAGL6* had effect on the expression of some genes central to sepal development.

Silencing of *SlAGL6* generates shorter, light green petals with altered epidermis cells

Usually, the normal petals of WT flowers at anthesis are yellow, but in transgenic flowers, the color of petals was more green than wild type at the same time (Fig. 4a, b). Scanning electron microscopy analysis revealed the adaxial epidermis cells which were protruding and clear-cut in WT petals (Fig. 4c) converted into cave-shape and fuzzy-outline cells in transgenic lines (Fig. 4d) at 2 dpa. At the same time, we measured the petal length and found that transgenic petals were shorter than WT (Fig. 4e).

Total chlorophyll content increases and chlorophyll synthesis genes of transgenic plant are up-regulated

To further confirm the phenotype of green petal in transgenic flowers, we measured the content of total chlorophyll in petals of WT and transgenic lines. The content of total chlorophyll in transgenic petals rose to 1.3–1.8-fold compared with WT at 2 dpa (Fig. 5a). At the same time, chlorophyll synthesis related genes were also detected by quantitative real-time PCR. The chlorophyll biosynthesis genes, *SlGLK1* and *SlGLK2* (Powell et al. 2012) were significantly up-regulated (*SlGLK1* to 2.8-–fourfolds and *SlGLK2* to 1.8–3.6-folds) in petals of transgenic lines (Fig. 5b, c). Similarly, the expression of two photosystem-related genes also increased (*SlCab-7* to 1.2-–twofolds and *SlrbcS3B* to 1.9–3.2-folds) (Fig. 5d, e).

Transcripts of tomato B-class floral organ identity genes in petals are reduced

Since the *SlAGL6* was highly expressed in petals and repressing of *SlAGL6* caused the conversion of color and cellular morphology, we detected the expression of four reported tomato B-class genes *TAP3* (Kramer et al. 1998; Quinet et al. 2014), *TPI* (Geuten and Irish 2010; Kim et al. 2005), *TM6* (Busi et al. 2003; Sung et al. 2000) and *SlGLO1* (Geuten and Irish 2010; Guo et al. 2016) in petals of WT and transgenic lines. For *TPI*, the transcript accumulation in transgenic lines remained nearly at the same level as WT except in line RNAi-2 (Supplementary Fig. 2c). However, the expression of *TM6, TAP3* and *SlGLO1* decreased a little in transgenic lines (Fig. 6). The transcription of *MC* in transgenic petals was also examined and except for RNAi-2, other lines showed no significant difference with WT (Supplementary Fig. 2b). The results illustrated that *SlAGL6* moderately affected partial B-class genes and had no influence on A-class genes in petals.

Expression of C, D and E-class genes were affected in transgenic lines

Although no obviously difference was observed in stamens and carpels in *SlAGL6* transgenic lines, we still detected the transcription of C-class gene *TAG1* and D-class gene AGL11 in stamens and carpels. As supplementary, two E-class genes, *TAGL2* and *TM5* were detected in each floral organ. Results showed that in stamen and carpel, *TAG1* had no significant difference in stamen between WT and transgenic lines while in carpel, the expression of *TAG1* reduced a little in two transgenic lines (Supplementary Fig. 2d). *AGL11* was repressed in both stamen and carpel (Supplementary Fig. 2e). The expression of *TAGL2* had no difference between WT and transgenic lines in petals and

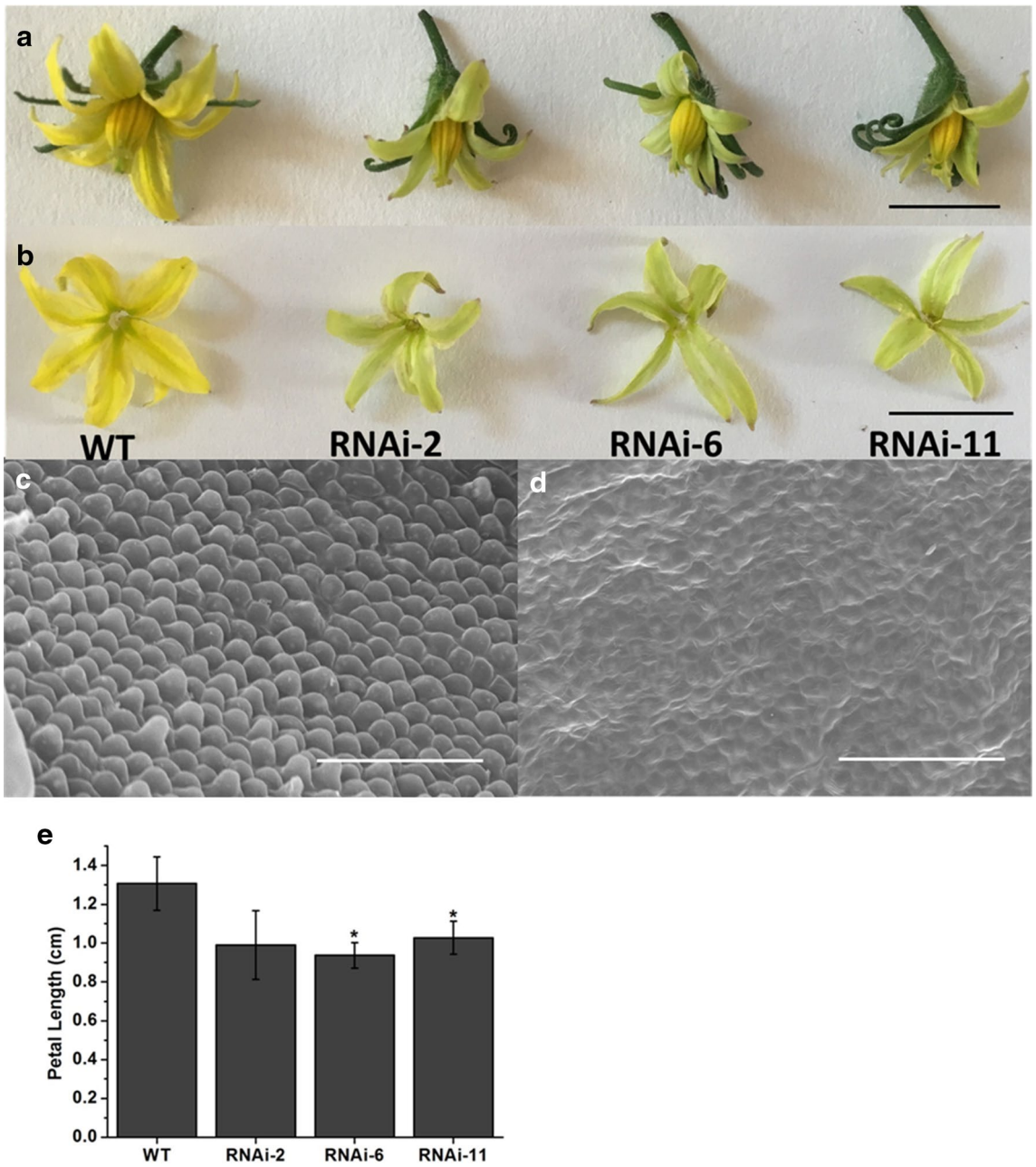

Fig. 4 Phenotype of petal in SlAGL6 RNAi lines. **a** Flowers of WT and transgenic lines at 2 dpa. *Bars* 1 cm. **b** Separated petals of WT and transgenic lines. *Bars* 1 cm. Surface of adaxial petal of WT (**c**) and transgenic (**d**) flower. *Bars* 60 μm. **e** Petal length of WT and transgenic lines. *Error bars* represent the standard error of the mean ($n = 10$). *Asterisks* indicate a significant difference ($P < 0.05$) between WT and transgenic lines

stamens, while in sepals, the transcription of *TAGL2* was repressed in only one transgenic line. It is worth noting that the expression of *TAGL2* was down-regulated remarkably in carpels (Supplementary Fig. 2f). The transcription of *TM5* raised in two transgenic lines in petals and elevated in carpels obviously. The expression of *TM5* in stamens was barely affected while in sepals, it was only affected in RNAi-2 (Supplementary Fig. 2g).

Discussion

In previous research, it was extensively accepted that *AGL6* linage was active in specifying the identity of the floral meristem, determining the flowering time and development of floral organs. But the function of *AGL6* is still not completely clarified. In some basal angiosperms, *AGL6* lineage genes have been considered to be A-function genes for their

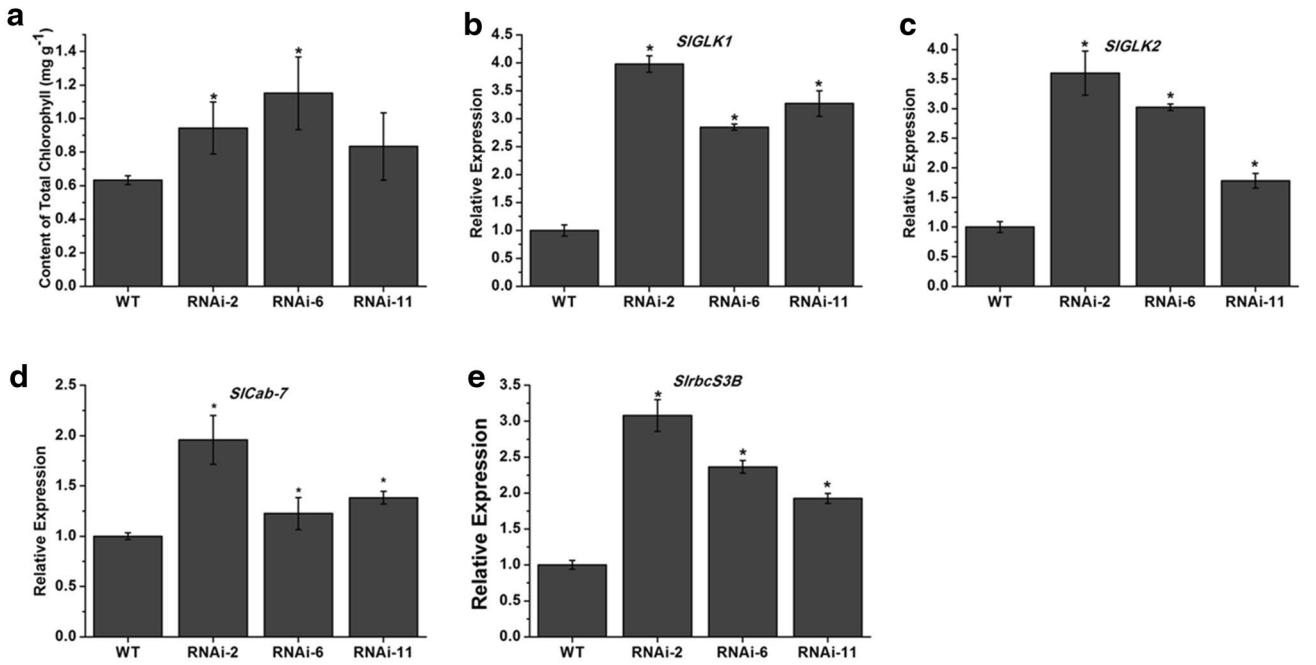

Fig. 5 Total chlorophyll content and detection of chlorophyll synthesis genes in petal of WT and transgenic lines. **a** Total chlorophyll content of petals from WT and transgenic flowers. **b–d** Expression analysis of *SlGLK1, SlGLK2, SlCab-7* and *SlrbcS3B* in petals of WT and transgenic lines at 2 dpa. Each value represents the mean ± SE of three replicates. *Asterisks* indicate a significant difference ($P < 0.05$) between WT and transgenic lines

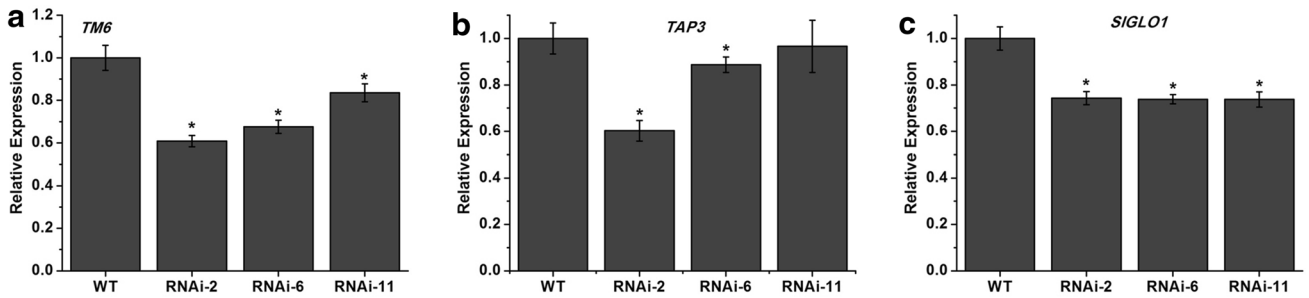

Fig. 6 Expression analysis of B-class genes in petal of WT and transgenic lines. Genes were detected in petals of WT and transgenic lines at 2 dpa. Each value represents the mean ± SE of three replicates. *Asterisks* indicate a significant difference ($P < 0.05$) between WT and transgenic lines

high expression in sepals (Chanderbali et al. 2006; Kim et al. 2005; Wang et al. 2016) and floral induction function by affecting flowering time (Hsu et al. 2003; Wang et al. 2011). However, in *Petunia, PhAGL6* was proved to be more similar to *SEP* (E-function) than to *AP1* (A-function) genes in both sequence and expression pattern (Rijpkema et al. 2009). *Arabidopsis AGL6* lineage member, *AGL13* was claimed to be a putative ancestor for the E-functional genes (Hsu et al. 2014). In this study, expression pattern showed that tomato *AGL6* lineage member *SlAGL6* expressed highly in flowers and four whorls organ expression profile revealed that the highest expression of *SlAGL6* located in petals and carpels, while the expression in sepals was relatively lower. The expression profile was similar to *Petunia AGL6* lineage member *PhAGL6* whose expression resembled E-class gene *FBP2* (Rijpkema et al. 2009) suggesting *SlAGL6* is a potential E-function gene. Silencing S*lAGL6* caused abnormality in sepals and petals. While along with the development of flower, the transcript accumulation of *SlAGL6* maintained at a stable level indicated that *SlAGL6* is likely to play role in the whole development process of flowers.

In *SlAGL6* RNAi lines, we found that the number of flowers with fused sepals and fused sepals in a single flower were remarkably higher than in WT (Supplementary Table S3). The few layers of cells between sepals in

cross section of transgenic flowers (Fig. 2f) suggested that the formation of partial boundaries in the first whorl was probably influenced. A tomato MADS-box gene *MC* and two NAC-domain transcription factors *GOBLET* and *SlNAM2* were detected in sepals of transgenic lines and WT. *MC* is a well-known tomato A-function gene involving in sepal development (Vrebalov et al. 2002). Previous research showed that *SlNAM2* participates in the establishment of tomato flower whorl and sepal boundaries (Hendelman et al. 2013), while *GOBLET* is required to inhibit congenital fusion with primordia in the same whorl (Berger et al. 2009). Based on our results, we draw a conclusion that *SlAGL6* affects the development of sepals via A-function genes and leads to sepals fusion by influencing genes participating in boundary establishment.

Down-regulation of *SlAGL6* led to light green petals with increased total chlorophyll content and up-regulated chlorophyll biosynthesis genes *SlGLK1* and *SlGLK2*. In previous research, repression of tomato E-function genes *TM5* (Pnueli et al. 1994b) and *TAGL2* (*TM29*) (Ampomah-Dwamena et al. 2002), B-function genes *TPI* (Mazzucato et al. 2008) and *SlGLO1* (Guo et al. 2016) also produced green petals. What's more, the expression of *SlGLK1* and *SlGLK2* also increased in *SlGLO1* RNAi lines (Guo et al. 2016). Taken together, we proposed that *SlAGL6* affected the synthesis of chlorophyll in petals likely by influencing *SlGLO1*. In our study, expression of the two E-function genes *TAGL2* and *TM5* was also detected, results showed that *TAGL2* had no difference between WT and transgenic lines in petals, while *TM5* raised in two transgenic lines (Supplementary Fig. 2f, g). *SlCab-7* encodes the type LHCI Type II chlorophyll a/b-binding polypeptide of Photosystem I (Pichersky et al. 1988) and *Slrbcs3B* encodes the small subunit of ribulose-l, 5-bisphosphate carboxylase (Sugita et al. 1987). The repression of *SlAGL6* in petals of transgenic lines might maintain more active photosynthetic function than wild type for the increasing expression of *SlCab-7* and *Slrbcs3B*. Petals of *SlAGL6* RNAi lines also exhibited shorter size than WT and alteration in epidermis cells morphology. The same phenotype was also appeared in *Petunia*. Lack of function of *PhAGL6* produced petals with smaller size and conversed petal epidermis cells into cells similar to those found in sepal epidermis redundantly with *PhFBP2* and *PhFBP5* (Rijpkema et al. 2009). According to the former reports in tomato, repressing B-class genes *SlGLO1* and *TM6* generated shorter petals probably caused by a decrease in cell proliferation (de Martino et al. 2006; Guo et al. 2016). The alteration in morphology of epidermis cells in petal was observed in *tap3* homozygous mutant while in *SlGLO1* RNAi lines, the adaxial cells were more sparse than WT (de Martino et al. 2006; Guo et al. 2016). In *SlAGL6* transgenic lines, the expression of these genes reduced a little indicating that the same alteration may also appeared in *SlAGL6* transgenic lines. In this study, we indeed observed that the morphology of cells in transgenic lines altered. These results revealed that *SlAGL6* may function directly or indirectly by affecting B-class genes in petal development.

In this study, we did not observed any differences between WT and transgenic plants in stamen and carpel as well as flowering time, inflorescence structure and flower number under normal growth condition. But we also examined the expression of C-class gene *TAG1* and D-class gene *AGL11*. Repression of TAG1 displays homeotic conversion of stamens into petaloid organs and the replacement of carpels with pseudocarpels bearing indeterminate floral meristems with nested perianth flowers (Pnueli et al. 1994a). As a D-class member, tomato *AGL11* is responsible for seed development and silencing tomato *AGL11* produces seedless fruits (Ocarez and Mejia 2016). However, in our study, no obvious difference was observed in seed development although the expression of *AGL11* was down-regulated (Supplementary Fig. 2e), but recent study showed that *Slagl6* mutation set seedless fruits under heat stress condition (Klap et al. 2016) which indicating that repression of *SlAGL6* affects the expression of D-class gene and has impact on seed development in abnormal environment.

The regulatory network between MADS-box floral homeotic proteins is huge and complex based on DNA structure and combinatorial interactions (Yan et al. 2016). As an important floral homeotic gene, *AGL6* works by affecting the expression of other genes especially MADS-box genes. In *Petunia*, PhAGL6 was proved by yeast-two-hybrid assay to interact with SOC1-clade proteins, C- and D-function proteins and other SEP-clade proteins, however, it does not interact with B-function proteins (Rijpkema et al. 2009). In *Arabidopsis, AGL6* was confirmed to inhibit the transcription of *FLOWER LOCUS C*/*MADS AFFECTING FLOWERING* (*FLC*/*MAF*) genes and up-regulate *FLOWERING LOCUS T* (*FT*) to promote flowering (Yoo et al. 2011b). The other *Arabidopsis AGL6* lineage member *AGL13* was proved to regulate the expression of *AG*/*AP3*/*PI* by positive regulatory feedback loops and suppress its own expression through negative regulatory feedback loops by acting as a repressor, *AGL6* (Hsu et al. 2014). Ectopic expressing Orchid *AGL6*-like gene *OMADS1* in *Arabidopsis* changed the expression of *FT, SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) and flower meristem identity genes *LEAFY* (*LFY*) and *AP1* (Hsu et al. 2003). In rice, the transcript levels of A-, B-, C- and E-class genes were affected in mutant of *AGL6* lineage gene *MADS6* (Li et al. 2011; Ohmori et al. 2009). In this study, A-class gene *MC* was only reduced in sepals of transgenic lines (Fig. 3a) and three B-function genes *TM6, TAP3* and *SlGLO1*were down-regulated in petals of transgenic lines (Fig. 6). The detection of

MADS-box genes in RNAi flower suggested that *SlAG6* participates in the development of tomato sepals by regulating A-class genes and petals by regulating B-class genes. In *Petunia* and rice, *AGL6* genes were all testified to function redundantly with other *AGL6* members or E-function genes and lead to a minor phenotype in single mutant or RNAi plants (Ohmori et al. 2009; Rijpkema et al. 2009). In tomato, by sequence alignment, only one member of the *AGL6* lineage was identified. The phenotype appeared in *SlAGL6* transgenic lines was quite feeble, so whether redundancy with genes in other classes exists in the functioning process of *SlAGL6* is still a question to be solved.

In summary, *SlAGL6* participates in the development of tomato sepals and petals. So far, the functions of AGL6 lineage members in *Arabidopsis*, rice, *Petunia* and tomato have been studied and there are both similarities and differences among different species. *AGL6* lineage is an ancient clade in MADS transcription factor and focusing on the functional study of *AGL6* lineage members in different species will help us get better acquaintance about evolution and functional divergence of MADS-box genes.

Author contribution statement GC and ZH designed and managed the research work and improved the manuscript. XY, XG, YL, JZ, JH and ST performed the experiments. XY wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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