First posted online on 20 September 2019 as 10.1242/dev.177527
Access the most recent version at http://dev.biologists.org/lookup/doi/10.1242/dev.177527

Wheat AGAMOUS LIKE 6 transcription factors function in stamen development by regulating the expression of *Ta APETALA3*

Yali Su[1,#], Jinxing Liu[2,#], WanqiLiang[3], YanhuaDou[1], RuifengFu[3], Wenqiang Li[1], Cuizhu Feng[1], Caixia Gao[2], Dabing Zhang[3], Zhensheng Kang[4,*], HaifengLi[1,*]

[1]State Key Laboratory of Crop Stress Biology for Arid Areas and College of Agronomy, Northwest A&F University, Yangling 712100, China

[2]Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China

[3]School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

[4]State Key Laboratory of Crop Stress Biology for Arid Areas and College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, 712100, China

[#] These authors contributed equally to this work.

*Corresponding Author: Haifeng Li, lhf@nwsuaf.edu.cn; Zhensheng Kang, kangzs@nwsuaf.edu.cn

Key words: Wheat, *AGAMOUS LIKE 6*, stamen, *APETALA3*, MADS-box, flower

Summary statement: In wheat, *AGAMOUS LIKE 6* genes function as E-class genes and play essential roles in stamen development through transcriptional regulation of *Ta APETALA3*.

Abstract

Previous studies have revealed the functions of rice and maize *AGAMOUS LIKE 6* (*AGL6*) genes *OsMADS6* and *ZAG3*, respectively, in floral development; however, the functions of three wheat (*Triticum aestivum*) *AGL6* genes are still unclear. Here, we have reported the main functions of wheat *AGL6* homoeologous genes in stamen development. In RNAi plants, stamens showed abnormality in number, morphology, and a tendency to transform into carpels. Consistently, the expression of B-class gene *TaAPETALA3* (*AP3*) and auxin-responsive gene *TaMGH3* was down-regulated, whereas the wheat ortholog of the rice carpel identity gene *DROOPING LEAF* was ectopically expressed in RNAi stamens. TaAGL6 proteins bind to the promoter of *TaAP3* directly. Yeast one hybrid and transient expression assays further showed that TaAGL6 positively regulate the expression of *TaAP3 in vivo*. Wheat AGL6 transcription factors interact with TaAP3, TaAGAMOUS and TaMADS13. Our findings indicate that TaAGL6 transcription factors play an essential role in stamen development through transcriptional regulation of *TaAP3* and other related genes. We have proposed a model to illustrate the function and probable mechanism. This study extends our understanding of *AGL6* genes.

INTRODUCTION

Flower development is the basis for seed development in angiosperms. On the basis of analyses of flower mutants in the model dicot species *Arabidopsis thaliana* and *Antirrhinum majus*, the ABCDE model was proposed to interpret the molecular mechanism underlying flower development (Coen et al., 1991; Pelaz et al., 2000; Theissen, 2001; Ditta et al., 2004).

In rice (*Oryza sativa*), the functions of many floral genes have been elucidated. These genes included *OsMADS14*, *OsMADS15* and *OsMADS18* (Kobayashi et al., 2012; Wu et al., 2017), B class gene *OsMADS16/SPW1* (Nagasawa et al., 2003), C class genes *OsMADS3* and *OsMADS58* (Yamaguchi et al., 2006; Dreni et al., 2011; Hu et al., 2011) , D class gene *OsMADS13* (Dreni et al., 2007; Li et al., 2011b), E class genes *OsMADS7*, *OsMADS8* (Cui et al., 2010), *OsMADS1* (Jeon et al., 2000), *OsMADS34* (Gao et al., 2010; Kobayashi et al., 2010; Lin et al., 2014), and carpel identity gene *DROOPING LEAF* (*DL*) (Yamaguchi et al., 2004; Li et al., 2011b).

In *MADS-box* gene family, *AGAMOUS LIKE 6* (*AGL6*) genes constitute an ancient sub-family of *MADS-box* genes and can be found extensively in the plant kingdom (Becker et al., 2003; Reinheimer et al., 2009).

Two *AGL6* genes have been identified in *Arabidopsis*: *AGL6* and *AGL13*. Because of probable functional redundancy, null mutant plants of *agl6-2* showed no obvious phenotypes in flowering time and floral development (Koo et al., 2010). Analyses of transgenic *Arabidopsis* over-expressing *AGL6* showed its function in flowering time (Koo et al., 2010; Yoo et al., 2011b). Additionally, *AGL6* functions in leaf movement and is involved in the formation of the axillary bud (Yoo et al., 2011a; Huang et al., 2012). *AGL13* regulates the development of male and female gametophytes (Hsu et al., 2014).

In *Petunia*, *pMADS4*/*PhAGL6* showed redundant functions with E class genes *FBP2* and *FBP5* (Rijpkema et al., 2009).

In grasses, maize (*Zea mays*), *AGL6* gene *ZAG3* regulates the development of floral organs and meristems (Thompson et al., 2009). Rice *OsMADS6* affects the development of paleas, lodicules, stamens, and ovules as well as the floral meristems (FMs) and seeds (Ohmori et al., 2009; Li et al., 2010; Zhang et al., 2010; Duan et al., 2012). Analyses of genetic interactions between *OsMADS6* and other floral genes indicated that *OsMADS6* is a master floral regulator (Li et al., 2011a).

The bread wheat (*Triticum aestivum*) is a hexaploid plant (Shewry, 2009). Although an ABCDE model was proposed for wheat (Murai, 2013), the biological functions of most wheat floral genes have not yet been elucidated.

Previous study showed *TaMADS12* and *TaAGL37* are *AGL6* homologues in wheat (Paolacci et al., 2007; Reinheimer et al., 2009). In this study, we investigated the functions of wheat *AGL6* homoeologous genes. Our results showed that TaAGL6 transcription factors play an essential role in stamen development through the transcriptional regulation of *TaAP3*.

RESULTS AND DISCUSSION

Wheat genome has three *AGL6* homoeologous genes

For the sake of cloning the full cDNAs of *TaAGL6*, 3′ and 5′ rapid amplification of cDNA Ends (RACE) experiments were performed (Table S1). About 1000 bp and 400 bp PCR products in which 205 bp overlapped were obtained (Fig. S1A), and they were cloned into T vectors. Sequencing results showed that both 3′ and 5′ RACE products included three conserved but distinctive sequences (Fig. S1B), indicating that there are three homoeologous *TaAGL6* genes, which is consistent with the wheat genome (Appels et al., 2018). According to chromosome locations, they were named as *TaAGL6-A*, *TaAGL6-B*, and *TaAGL6-D*. *TaAGL6-A* is corresponding to reported *TaMADS37*, and *TaAGL6-B* is corresponding to *TaMADS12*. Every cDNA includes an open reading frame and encodes one AGL6 protein (Fig. S2). In addition to MADS-box and K domains, these proteins have AGL6 motifs I and II (Fig. S2). They were classified into the grass AGL6-I-ZAG subgroup (Dreni et al., 2016). Although some SNPs resulted in some differences in the protein sequence, but they do not affect the functions seriously, according to the prediction with the software Protein Variation Effect Analyzer (Fig. S2B). These results indicated that they have similar functions.

TaAGL6 genes display conserved expression patterns

Firstly, we analyzed their expression pattern by using quantitative RT-PCR with primers simultaneously applicable for *TaAGL6-A*, *TaAGL6-B*, and *TaAGL6-D*. Consistent with previous report (Feng et al., 2017), results showed that *TaAGL6* genes are strongly expressed in the inflorescences from stage 3-8.5 (Fig. 1A and B), according to previously classification (Waddington et al., 1983). The transcripts mainly accumulate in the paleas, lodicules, and pistils at stage 6-7. Relatively, the expression in the lemmas and stamens is very low (Fig. 1C). In addition, qRT-PCR with specific primers showed that these three *TaAGL6* genes have similar expression patterns in the floral organs (Fig. S3).

We further performed in situ hybridization. The signal was not detected in the inflorescences at stage 2 (Fig. 1D), and the transcripts firstly emerged in the FMs at stage 3 (Fig. 1E) and then in the palea and lodicule primordial at stage 3.5-4 (Fig. 1F and G). Then, signals were continually detected in the developing lodicules and paleas, at stage 4-7.5 (Fig. 1H-P).

Meanwhile, the transcripts were detected in the carpels and ovules at stage 4.5-7.5 (Fig. 1I–P), while no obvious signal was found in the lemmas and stamens (Fig. 1I, K–P), because of the low level expression. When hybridized with sense probes, no signal was found (Fig.1Q).

Over-expressing *TaAGL6* genes promotes early flowering

TaAGL6 genes were over-expressed in *Arabidopsis* respectively. Generally, the transgenic plants harboring different *TaAGL6* genes showed similar phenotypes that could be classified into two types: early flowering and dwarf (Fig. S4A–E), and increased number of stems or branches (Fig. S4F–J). Relatively, the expression level of the target gene was higher in the early flowering lines than in the multi-branch lines (Fig. S5A). These observations further indicate that three *TaALG6* genes have similar functions.

FLOWERING LOCUS T (*FT*) in *Arabidopsis* is a master flowering regulator (Gu et al., 2013), we analyzed the expression of *FT* in our transgenic plants. The expression level of *FT* is higher in the transgenic lines than the control during the vegetative stage, consistent with the early-flowering phenotype (Fig. S4K).

We generated transgenic wheat overexpressing *TaAGL6-B* too and obtained 27 positive lines. The expression level of the target gene is increased in most lines, especially in lines 18 and 30 (Fig. S5B). The flowering time of 12 lines was 10–20 days earlier than that of the control (Fig. S6A–E), and the expression of wheat *FT* homologs was up-regulated in the transgenic wheat (Fig. S6F). But no floral phenotype was observed.

***TaAGL6* genes functions in stamen development**

Because all three *TaAGL6* genes are expressed in the flowers and showed similar functions in transgenic *Arabidopsis*, we speculated that their functions are redundant. Therefore, we knock down these *TaAGL6* genes simultaneously by using RNA interference (RNAi). A total of 21 transgenic wheat lines were obtained. qRT-PCR results showed that the expression of *TaAGL6* genes in lines 17-2, 28-3 and 3-2 is decreased drastically (Fig. S5C), so they were selected for further analyses. At the vegetative stage, none of them showed obvious phenotypes. At the reproductive stage, abnormal floral phenotypes were observed.

In about 30% RNAi florets, stamens display abnormality in the number or/and the morphology. One normal floret generates three stamens (Fig. 2A), while some transgenic florets only bear two stamens (Fig. 2B). The morphology of some RNAi stamens were also affected (Fig. 2C and D). As shown in Fig. 2E, a normal stamen has four anther cavities; however, in some transgenic stamens, two anther cavities fuse together or one stamen only has two anther cavities (Fig. 2F and G). The vigor of pollen grains was affected in all RNAi stamens, irrespective of morphology (Fig. 2H–J). As a result, the seed-setting rate is lower

(35.81%) than the control (96.34%) (Fig. 2K). Occasionally, some florets displayed strong phenotypes by generating three or four carpels without stamens or with anther-stigma mosaic organs (Fig. 2L–N).

Scanning electron microscope (SEM) observation showed the developmental defects of stamens at the early stage, which is consistent with these phenotypes. In normal cases, three stamen primordia are formed; however, in some transgenic florets, only two normal stamen primordia were observed (Fig. 2O and P). In addition, all stamens in the wild type are quadrangular; however, in some transgenic florets, only one stamen was quadrangular and the morphology of the other two stamens was not normal (Fig. 2Q and R). These phenotypes were observed in not only T_0 plants but also T_1 and T_2 plants.

Since only a single *AGL6* gene was found in the wheat A, B, and D sub-genome and these homoeologous genes are mainly expressed in the paleas and lodicules, we predicted that wheat *AGL6* genes function as rice OsMADS6 genes and play roles in paleas and other organs as well.

Surprisingly, no obvious phenotype was observed in RNAi paleas and lodicules. Reinheimer and Kellogg (2009) proposed that the expression of grass *AGL6* genes in the ovule is conserved, whereas the expression domain of the palea is acquired during the evolution process. Maybe *TaAGL6* genes acquire the expression domain in the paleas, but they have no functions in palea development. During the evolution process, the functions of *AGL6* become divergent, and *OsMADS6* gains the function in specifying palea identity. Another possibility is the low expression of TaAGL6 genes in RNAi lines is enough to maintain normal palea and lodicules development.

TaAGL6 transcription factors interact with other floral regulators

Several AGL6 transcription factors interact with floral regulators as. ZAG3 forms a heterodimer with C-function protein ZAG1 (Thompson et al., 2009); OsMADS6 interacts with D-function protein OsMADS13 (Li et al., 2011a). OsMADS6 and OsMADS17 form protein complexes with B-function proteins (Seok et al., 2010). Similar to E- function protein FBP2, petunia AGL6 interacts with C- and D-function proteins (Rijpkema et al., 2009). These and other results indicate that AGL6 transcription factors have partial E- functions proteins.

To clarify whether TaAGL6 proteins interact with other floral regulators, yeast two hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays were performed. The results showed that TaAGL6-A interact with TaAP3, TaAG, and TaMADS13 not only in yeast cells, but also in tobacco (*Nicotiana benthamiana*) leaf cells (Fig. 3A and 3B). We further

verified these interactions in *planta* by performing Co-Immunoprecipitation (Co-IP) (Fig. 3C). In addition, TaAGL6-A displayed transcription activity, whereas TaAP3, TaAG, and TaMADS13 did not (Fig. S7). Meanwhile, TaAGL6-B and TaAGL6-D interact with TaAP3, TaAG, and TaMADS13 in yeast cells and tobacco leaf cells (Fig. S8). These results showed that TaAGL6 proteins act as E-class transcription factors, and further implied redundant functions.

TaAGL6 transcription factors regulate stamen development by targeting *TaAP3* directly

Previously, we investigated the expression patterns of *TaAP3*, *TaAG*, *TaDL*, *TaMADS13*, *TaSEP*, and *TaLHS1* in floral organs (Li et al., 2016). We analyzed the expression of related genes in *TaAGL6* RNAi stamens. qRT-PCR results showed that the expression of *TaAP3* and *TaMGH3* is obviously decreased in RNAi stamens. The ectopic expression of *TaDL*, *TaMADS13*, and *TaLHS1* was detected, whereas the expression of *TaAG* and *TaMADS58* is slightly increased in the RNAi stamens (Fig. S9). These results further verified the developmental defects in *TaAGL6* RNAi stamens at the molecular level.

OsMGH3 is an auxin-responsive gene and regulated by *OsMADS6* indirectly, and it functions in flower development (Zhang et al., 2010; Yadav et al., 2011). We screened the promoters of three *TaMGH3* genes, no CArG motif was found. Therefore, they are indirect downstream genes of *TaAGL6* genes. In wheat, two *TaAP3* homoeologous genes exist: *TaAP3-B* and *TaAP3-D*. In the promoters of *TaAP3-B* and *TaAP3-D*, two CArG motifs were found, respectively (Fig. 4A). Taking the transcription activity of TaAGL6 proteins into account, we wondered if TaAGL6 transcription factors regulate the expression of *TaAP3* directly. Therefore, we expressed and purified the TaAGL6-B-GST fusion protein (Fig. 4B and C) for electrophoresis mobility shift assay (EMSA). The EMSA results showed that the fusion protein could bind to motif 1 (Fig. 4D), but it could not bind to the other three motifs (Supplemental Fig. S10). Results of yeast one hybrid (Y1H) further verified the interactions between three TaAGL6 transcription factors and M1 (Fig.4E and F). Transient expression assay further showed the positive regulation of TaAGL6 to *TaAP3-B* in *planta* (Fig 4G and H).

As transcription factors, MADS-box proteins function through transcriptional regulation of target genes. In addition to regulating the expression of *OsMGH3* indirectly (Zhang et al., 2010), *OsMADS6* regulates the expression of *OsMADS58* and *OsFDML1* directly (Li et al.,

2011b; Tao et al., 2018). However, we failed to identify the ortholog of *OsFDML1* in wheat genome (data not shown). These results indicate that the detailed mechanism is different.

Mutations of *AP3* resulted in the homeotic transformation of stamens to carpels. Similar phenotypes were observed in *Antirrhinum majus defa*, maize *Silky1*, and rice *spw1* mutant plants (Schwarz-Sommer et al., 1990; Ambrose et al., 2000; Nagasawa et al., 2003; Whipple et al., 2004), suggesting important and conserved functions of *AP3* genes during stamen development.

The disrupted expression of *AP3* in *TaAGL6* RNAi stamens may be the cause of abnormality in stamen development (Fig. 2). In addition, *SPW1* represses the expression of *DL* (Nagasawa et al., 2003). Maybe this mechanism is conserved in wheat (Murai, 2013). Consistent with this prediction, *TaDL* is ectopically expressed in *TaAGL6* RNAi stamens, accompanied with deceased expression of *TaAP3* (Fig. S9), indicating the potential homeotic transformation from stamens to carpels. The ectopic expression of *TaLHS1* and *TaMADS13* (Fig. S9) and the severe phenotypes (Fig. 2) further support this prediction.

In general, our results extended understanding of *AGL6* genes and provided further evidence that AGL6 proteins have E- functions. Moreover, our findings indicate the distinctive function and mechanism of *TaAGL6* genes in stamen development. We have proposed a model to illustrate these findings (Figure S10). Further studies will help us to reveal the mechanism.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wheat varieties Chinese Spring (CS) and Kenong199 (KN199) were used in this study. They were planted in a glasshouse under a photoperiod of 12 h of light at 22°C and 12 h of darkness at 16°C or in an isolated experimental field under natural conditions at thr Northwest A&F University (108°4′E, 34°15′N). The *Arabidopsis* ecotype Columbia-0 (Col-0) was used to generate the transgenic lines in growth chambers with a light period of 16 h and dark period of 8 h at 23°C. Phenotypes were observed in the T2 and T3 plants. Tobacco was planted under the same growth conditions as those of *Arabidopsis*.

RACE

Total RNA was extracted from CS flowers, and the SMART RACE kit (TaKaRa) was used to synthesize the cDNA and generate the 5′-RACE and 3′-RACE products, according to the protocol. The specific primers for 5′-RACE (5GSP in Supplemental Table S1) and 3′-RACE

(3GSP in Supplemental Table S1) were designed by Primer Premier 5.0. Specific PCR products were cloned into the pMD-19T vector (TaKaRa), and different positive clones were sequenced.

Quantitative RT-PCR

The roots, stems, leaves, and flowers of wheat were collected at the heading stage. Inflorescences at different stages were collected and judged according to a previous study (Waddington et al., 1983). Glumes and different floral organs were collected from florets at stage 6-7. Total RNAs were extracted from different organs by using the TRIzol reagent (Sangon Biotech), according to the manufacturer's instructions. cDNAs were synthesized with AMV reverse transcriptase (Roche). qRT-PCR was performed using TB Green Premix Ex TaqII (TaKaRa) and CFX96 real-time PCR detection system (Bio-Rad). Three technical and three biological replicates were used. The data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak et al., 2001). The common primers were designed according to the consensus sequences, while the specific primers (TaAGL6AF/ TaAGL6AR, TaAGL6BF/ TaAGL6BR, and TaAGL6DF/ TaAGL6DR) for analyzing the expression of every *TaAGL6* gene were designed according to the differences between the full cDNA sequences, especially 5-bp insertion in 5′ UTR of *TaAGL6-A*, and 9- bp deletion in 3′UTR of *TaAGL6-D*. The specificity of each pair primers was verified by sequencing the RT-PCR products (data not shown). The premiers are listed in Table S1.

In Situ Hybridization

CS inflorescences at different stages were fixed in FAA (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) overnight at 4°C. Then, the materials were treated and the experiments were performed as described previously (Li et al., 2011b; Tao et al., 2018). The probes labeled with digoxigenin were prepared using the in vitro transcription kit (Roche), according to the protocol and previous studies (Li et al., 2011b; Tao et al., 2018). The probes were amplified with primers TaAGL6RNAiPF/TaAGL6T7-R (henceforth referred to as the "antisense probe") and TaAGL6T7-F/TaAGL6RNAiPR (henceforth referred to as the "sense probe") (Supplemental Table S1).

Generating transgenic plants

To construct the over-expression vectors, one pair primers TaAGL6OF/ TaAGL6OR (Table S1) were designed to amplify the coding region, because of the identical sequence after the ATG and before the TGA in three genes (Fig. S1). Then the PCR products were clone in to pMD18-T vectors, and three different cDNAs were selected by sequencing different clones, and the encoding *TaAGL6* cDNAs were cloned into the pCAMBIA1301 vectors (TaKaRa)

with *Nco*I and *Bgl*II sites under the control of the 35S promoter, respectively. The dsRNAi vector was constructed according to a previous study (Li et al., 2010). Wheat was transformed via gene gun bombardment, according to a previous study (Zhang et al., 2015). Transformation of *Arabidopsis* was mediated by *Agrobacterium tumefaciens* GV3101.

Histological Analyses and Scanning Electron Microscopy

The materials were fixed in FAA at 4°C overnight, dehydrated in graded ethanol, and substituted by xylene. Then, they were embedded in Paraplast Plus (Sigma-Aldrich) and cut into 8 μm sections, which were stained with 0.2% toluidine blue and photographed using a Nikon E600 microscope and Nikon DXM1200 digital camera. Scanning electron microscopy (SEM) images were obtained using a JSM-6360LV (JEOL) scanning electron microscope, as described previously (Liu et al., 2016).

Y2H Assay

Y2H assays were performed using the GAL4-based two-hybrid system (Clontech). Self-activation assays of three TaAGL6, TaAP3, TaAG, and TaMADS13 were performed according to the protocol. The coding sequence (CDS) of three *TaAGL6* genes was cloned in the frame of pGADT7-Rec to generate pGAD-Preys. The coding regions of *TaAP3*, *TaAG* and *TaMADS13* were cloned in the frame of pGBKT7 to generate pGBK-Baits. The different combinations with pGAD-Preys and pGBK-Baits were co-transformed into Y2HGold yeast cells (Clontech). Positive clones on SD/-Trp/-Leu solid medium were transferred to SD/-Trp-Leu-His or SD/-Trp-Leu-His-Ade liquid medium to detect the interactions. Clones harboring pGADT7-*TaAGL6* and pGBKT7 were used as the negative control. The primers were listed in Table S1.

BiFC Assay

BiFC vectors pSPYNE and pSPYCE were used to construct nYFP-TaAGL6-A/B/D, TaAP3-cYFP, TaAG-cYFP, and TaMADS13-Cyfp, with the one step cloning kit (Yeasen Biotech). *Agrobacterium* GV3101 cells harboring different nYFP-TaAGL6 and different cYFP constructs were infiltrated into *N. benthamiana* leaves. YFP fluorescence was observed under a confocal laser scanning microscope (Olympus FV10 ASW). Three independent *N. benthamiana* leaves were observed for the analysis of every interaction.

Protein Expression and Purification and Western Blot Analysis

The partial cDNA (1-507 bp) of *TaAGL6-B* was amplified with the primers TaAGL6-BGSTF and TaAGL6-BGSTR (Table S1), and inserted into *Eco*RI and *Xho*I sites of the pGEX-6p-1 vector to express the fusion protein glutathione-*S*-transferase (GST)-TaAGL6-B. Then, the construct was introduced into BL21 (DE3) pLysS *E. coli* cells.

Positive clones were cultured in Luria-Bertani (LB) medium containing 50 mg/mL ampicillin at 37°C to OD_{600} = 0.6. Expression of the fusion protein was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside. The fusion proteins were purified using glutathione-agarose beads (Sangon Biotech), according to the manufacturer's instructions.

The purified proteins were separated on 12% SDS-PAGE gels and transferred to a polyvinylidene difluoride (0.45 μm) membrane (Millipore, cat: IPVH00010F). After blocking for 3 h in Tris-buffered saline Tween (TBST) with 5% nonfat milk, the membrane was washed with TBST three times (5 min each time) and incubated with the GST antibody at a dilution of 1:1,000 (D190101, Sangon Biotech) at 4°C overnight. Then, secondary goat anti-mouse IgG conjugated with horseradish peroxidase (D110087, Sangon Biotech) was incubated for 2 h at a dilution of 1:5,000. The target protein bands were visualized using a chemiluminescence reagent (Beyotime Biotechnology) (Song et al., 2018).

Electrophoretic Mobility Shift Assay

The fragments including the CArG cis-element in the *TaAP3* promoter were amplified using PCR with biotin-labeled or non-labeled primers (Supplemental Table S1). The biotin-labeled DNA probes were incubated with the TaAGL6-B-GST protein, and competing experiments were performed by adding excess non-labeled probes. Biotin-labeled probes incubated with GST protein served as the negative control. EMSA assay was performed using the LightShift Chemiluminescent EMSA Kit (20148, Thermo), according to the manufacturer's instructions. Briefly, the reaction mixture (20 μL: 10 μg purified fused protein or GST, 100 fmol biotin end-labeled probes, 2 μL 10× binding buffer, 1 μL of 1 μg/μL Poly (dI·dC), 1 μL 50% glycerol, 1 μL 1% NP-40, 1 μL of 1 M KCl, 1 μL of 100 mM $MgCl_2$, 0.5 μL of 200 mM EDTA, and double-distilled water) is incubated for 30 min at room temperature for the binding reaction and electrophoresed on a 10% native polyacrylamide gel. Then, the proteins are transferred to a nylon membrane (S4056, Millipore) in 0.5× TBE buffer at 380 mA for 45–60 min, and the binding between the proteins and biotin-labeled probes was detected by chemiluminescence (Feng et al., 2014).

Y1H Analysis

For the Y1H assay, three copies of the CArG element were cloned into the pAbAi vector to create the bait construct. The CDS of three *TaAGL6* genes were fused to the GAL4 AD in the pGADT7 vector to generate prey constructs, AD-*TaAGL6-A*, AD-*TaAGL6-B*, and AD-*TaAGL6-D*. The prey vector and the empty vector (AD), serving as the negative control,

were then transformed separately into yeast cells containing bait constructs, with the primers TaAP3F/TaAP3R and Mut TaAP3F/Mut TaAP3R (Table S1). The transformed yeast cells were diluted with a 10X dilution series and dotted on the SD plates lacking Leu and Ura with 200 ng/mL (optimized according to the protocol) Aureobasidin A. The binding between prey protein and bait sequence is judged by the growth of cells harboring bait construct and prey construct.

Transient expression assay

To generate reporter construct, a 1746-bp region upstream of TaAP3-B start codon was amplified and cloned into a pGreenII 0800-LUC vector (Hellens et al., 2005). To create effecter construct, TaAGL6-B CDS were cloned into pGreenII 62-SK vector (Hellens et al., 2005). The recombinant effecter and reporter plasmids were transfected into *A. tumefaciens* strain GV3101 (pGreenII series holding psoup plasmid) separately and co-infected into tobacco leaves. A dual-luciferase reporter assay system (Promega) was used to measure firefly LUC and renilla luciferase (REN) activities. The REN gene under the control of the CaMV 35S promoter and the LUC gene were in the pGreenII 0800-LUC vector (Hellens et al., 2005). Relative REN activity was used as an internal control, and LUC/REN ratios were calculated. At least three assay measurements were performed for each assay.

Co-IP assays

The HA-tag vector Gold and GFP-tag vector pCAMBIA1302 were used for Co-IP analysis. Full-length CDS of TaAGL6-B, TaAP3, TaAG, and TaMADS13 were amplified and inserted into the tag vectors. Agrobacterium strains carrying TaAP3-GFP, TaAG-GFP, or TaMADS13-GFP were co-infiltrated into tobacco leaves with an Agrobacterium strain carrying TaAGL6-B-HA and P19 silencer, respectively. After three days, leaves were harvested, frozen in liquid nitrogen, and homogenized with protein extraction buffer. Homogenates were centrifuged (13, 000 rpm) for 15 min at 4°C. The expressed HA-TaAGL6 and TaAP3-GFP, TaAG-GFP, TaMADS13-GFP, or GFP proteins, were immuno-precipitated with GFP-Trap MA beads (ChromoTek) at 4°C for 2 h. The immuno-precipitated proteins were washed four times with the lysis buffer and then eluted by boiling for 5 min with 2X loading buffer. Immunoblots were detected by performing Western blotting with an anti-GFP antibody (D190750-0100; Sangon; 1:1,000), anti-HA antibody (AH158; Beyotime; 1:1,000) and the secondary goat anti-mouse IgG conjugated with horseradish peroxidase (D110087, Sangon Biotech; 1:5000). Total proteins (50 mg) extracted from tobacco leaves co-expressing HA-TaAGL6 and TaAP3-GFP, TaAG-GFP, TaMADS13-GFP, or GFP were used as input

control, respectively (Li et al., 2019). Western blotting was performed as above.

Statistical Analyses

All statistical analyses were performed using Student's *t*-test in the Statistical Product and Service Solutions (SPSS) software. The statistic differences were examined using *t*-test, and the significance level was set at 0.05 ($P < 0.05$).

Accession Number

The gene number of *TaAGL6-A*, *TaAGL6-B*, and *TaAGL6-D* is *TraesCS6A02G259000*, *TraesCS6B02G286400*, and *TraesCS6D02G240200* in GRAMENE database (http://www.gramene.org/), respectively.

Acknowledgements

We appreciate the kind help from Dr. Dejun Han and Dr. Yu Wang in Northwest A&F University.

Competing interests

No competing interests declared.

Fundings

This work was supported by the National Natural Science Foundation of China (grant nos.31571657), the Program of Introducing Talents of Innovative Discipline to Universities (Project 111) from the State Administration of Foreign Experts Affairs (#B18042), and China Schorlarship Council (201806305041).

References

Ambrose B. A., Lerner D. R., Ciceri P., Padilla C. M., Yanofsky M. F., Schmidt R. J. (2000). Molecular and genetic analyses of the Silky1 gene reveal conservation in floral organ specification between eudicots and monocots. *Molecular Cell* **5:** 569-579.

Appels R., Eversole K., Feuillet C., Keller B., Rogers J., Stein N., Pozniak C. J., Stein N., Choulet F., Distelfeld A., et al. (2018). Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science* **361:** Science 361:eaar7191.

Becker A., Theissen G. (2003). The major clades of MADS-box genes and their role in the development and evolution of flowering plants. *Molecular Phylogenetics and Evolution* **29:** 464-489.

Coen E. S., Meyerowitz E. M. (1991). The war of the whorls: genetic interactions controlling flower development. *Nature* **353:** 31-37.

Cui R., Han J., Zhao S., Su K., Wu F., Du X., Xu Q., Chong K., Theissen G., Meng Z. (2010). Functional conservation and diversification of class E floral homeotic genes in rice (Oryza sativa). *Plant J* **61:** 767-781.

Ditta G., Pinyopich A., Robles P., Pelaz S., Yanofsky M. F. (2004). The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. *Curr Biol* **14:** 1935-1940.

Dreni L., Jacchia S., Fornara F., Fornari M., Ouwerkerk P. B., An G., Colombo L., Kater M. M. (2007). The D-lineage MADS-box gene OsMADS13 controls ovule identity in rice. *Plant J* **52:** 690-699.

Dreni L., Pilatone A., Yun D. P., Erreni S., Pajoro A., Caporali E., Zhang D. B., Kater M. M. (2011). Functional analysis of all AGAMOUS subfamily members in rice reveals their roles in reproductive organ identity determination and meristem determinacy. *Plant Cell* **23:** 2850-2863.

Dreni L., Zhang D. B. (2016). Flower development: the evolutionary history and functions of the AGL6 subfamily MADS-box genes. *Journal of Experimental Botany* **67:** 1625-1638.

Duan Y. L., Xing Z., Diao Z. J., Xu W. Y., Li S. P., Du X. Q., Wu G. H., Wang C. L., Lan T., Meng Z., et al. (2012). Characterization of Osmads6-5, a null allele, reveals that OsMADS6 is a critical regulator for early flower development in rice (Oryza sativa

L.). *Plant Molecular Biology* **80:** 429-442.

Feng C., Chen Y., Wang C., Kong Y., Wu W., Chen Y. (2014). Arabidopsis RAV1 transcription factor, phosphorylated by SnRK2 kinases, regulates the expression of ABI3, ABI4, and ABI5 during seed germination and early seedling development. *Plant J* **80:** 654-668.

Feng N., Song G., Guan J., Chen K., Jia M., Huang D., Wu J., Zhang L., Kong X., Geng S., et al. (2017). Transcriptome profiling of wheat inflorescence development from spikelet initiation to floral patterning identified stage-specific regulatory genes. *Plant Physiol* **174:** 1779-1794.

Gao X., Liang W., Yin C., Ji S., Wang H., Su X., Guo C., Kong H., Xue H., Zhang D. (2010). The SEPALLATA-like gene OsMADS34 is required for rice inflorescence and spikelet development. *Plant Physiol* **153:** 728-740.

Gu X., Wang Y., He Y. (2013). Photoperiodic regulation of flowering time through periodic histone deacetylation of the florigen gene FT. *PLoS Biol* **11:** e1001649.

Hellens R. P., Allan A. C., Friel E. N., Bolitho K., Grafton K., Templeton M. D., Karunairetnam S., Gleave A. P., Laing W. A. (2005). Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods*, **1**, 13.

Honma T., Goto K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature* **409:** 525-529.

Hsu W. H., Yeh T. J., Huang K. Y., Li J. Y., Chen H. Y., Yang C. H. (2014). AGAMOUS-LIKE13, a putative ancestor for the E functional genes, specifies male and female gametophyte morphogenesis. *Plant Journal* **77:** 1-15.

Hu L., Liang W., Yin C., Cui X., Zong J., Wang X., Hu J., Zhang D. (2011). Rice MADS3 regulates ROS homeostasis during late anther development. *Plant Cell* **23:** 515-533.

Huang X., Effgen S., Meyer R. C., Theres K., Koornneef M. (2012). Epistatic natural allelic variation reveals a function of AGAMOUS-LIKE6 in axillary bud formation in Arabidopsis. *Plant Cell* **24:** 2364-2379.

Ishikawa M., Ohmori Y., Tanaka W., Hirabayashi C., Murai K., Ogihara Y., Yamaguchi T., Hirano H. Y. (2009). The spatial expression patterns of DROOPING LEAF orthologs suggest a conserved function in grasses. *Genes Genet Syst* **84:** 137-146.

Jeon J., Jang S, Lee S, Jung K, Nam J, Kim C., Lee S, Chung Y, Kim S, Lee Y, et al. (2000). Leafy hull sterile 1 is a homeotic mutation in a rice MADS Box gene affecting rice flower development. *Plant Cell* **12:** 871-884.

Kobayashi K., Maekawa M., Miyao A., Hirochika H., Kyozuka J. (2010). PANICLE PHYTOMER2 (PAP2), encoding a SEPALLATA subfamily MADS-box protein, positively controls spikelet meristem identity in rice. *Plant Cell Physiol* **51:** 47-57.

Kobayashi K., Yasuno N., Sato Y., Yoda M., Yamazaki R., Kimizu M., Yoshida H., Nagamura Y., Kyozuka J. (2012). Inflorescence meristem identity in rice is specified by overlapping functions of three AP1/FUL-Like MADS Box genes and PAP2, a SEPALLATA MADS Box gene. *Plant Cell* **24:** 1848-1859.

Koo S., Bracko O., Park M., Schwab R., Chun H., Park K., Seo J., Grbic V., Balasubramanian S., Schmid M., et al. (2010). Control of lateral organ development and flowering time by the Arabidopsis thaliana MADS-box Gene AGAMOUS-LIKE6. *Plant Journal* **62:** 807-816.

Li D., Zhang H., Mou M., Chen Y., Xiang S., Chen L., Yu D. (2019). Arabidopsis class II TCP transcription factors integrate with the FT-FD module to control flowering. *Plant Physiol*, pii: pp.00252.2019.

Li H., Han Y., Liu M., Wang B., Su Y., Sun Q. (2016). Expression patterns of *MADS-box* genes related to flower development of wheat. *Acta Agron Sin* **42:** 1067-1073.

Li H., Liang W., Hu Y., Zhu L., Yin C., Xu J., Dreni L., Kater M. M., Zhang D. (2011a). Rice MADS6 interacts with the floral homeotic genes SUPERWOMAN1, MADS3, MADS58, MADS13, and DROOPING LEAF in specifying floral organ identities and meristem fate. *Plant Cell* **23:** 2536-2552.

Li H., Liang W., Jia R., Yin C., Zong J., Kong H., Zhang D. (2010). The AGL6-like gene OsMADS6 regulates floral organ and meristem identities in rice. *Cell Res* **20:** 299-313.

Li H., Liang W., Yin C., Zhu L., Zhang D. (2011b). Genetic interaction of OsMADS3, DROOPING LEAF, and OsMADS13 in specifying rice floral organ identities and meristem determinacy. *Plant Physiol* **156:** 263-274.

Lin X., Wu F., Du X., Shi X., Liu Y., Liu S., Hu Y., Theissen G., Meng Z. (2014). The pleiotropic SEPALLATA-like gene OsMADS34 reveals that the 'empty glumes' of rice (Oryza sativa) spikelets are in fact rudimentary lemmas. *New Phytol* **202:** 689-702.

Liu M., Li H., Su Y., Li W., Shi C. (2016). G1/ELE Functions in the Development of Rice Lemmas in Addition to Determining Identities of Empty Glumes. *Front Plant Sci* **7:** 1006.

Livak K. J., Schmittgen T. D. (2001). Analysis of relative gene expression data using

real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* **25:** 402-408.

Murai K. (2013). Homeotic Genes and the ABCDE Model for Floral Organ Formation in Wheat. *Plants (Basel)* **2:** 379-395.

Nagasawa N., Miyoshi M., Sano Y., Satoh H., Hirano H., Sakai H., Nagato Y. (2003). SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice. *Development* **130:** 705-718.

Ohmori S., Kimizu M., Sugita M., Miyao A., Hirochika H., Uchida E., Nagato Y., Yoshida H. (2009). MOSAIC FLORAL ORGANS1, an AGL6-like MADS box gene, regulates floral organ identity and meristem fate in rice. *Plant Cell* **21:** 3008-3025.

Paolacci A. R., Tanzarella O. A., Porceddu E., Varotto S., Ciaffi M. (2007). Molecular and phylogenetic analysis of MADS-box genes of MIKC type and chromosome location of SEP-like genes in wheat (Triticum aestivum L.). *Mol Genet Genomics* **278:** 689-708.

Pelaz S., Ditta G., Baumann E., Wisman E., Yanofsky M. (2000). B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature* **405:** 200-203.

Reinheimer R., Kellogg E. A. (2009). Evolution of AGL6-like MADS Box genes in Grasses (Poaceae): ovule expression is ancient and palea expression is new. *Plant Cell* **21:** 2591-2605.

Rijpkema A. S., Zethof J., Gerats T., Vandenbussche M. (2009). The petunia AGL6 gene has a SEPALLATA-like function in floral patterning. *Plant Journal* **60:** 1-9.

Schwarz-Sommer Z., Huijser P., Nacken W., Saedler H., Sommer H. (1990). Genetic control of flower development by homeotic genes in Antirrhinum majus. *Science* **250:** 931-936.

Seok H. Y., Park H. Y., Park J. I., Lee Y. M., Lee S. Y., An G., Moon Y. H. (2010). Rice ternary MADS protein complexes containing class B MADS heterodimer. *Biochem Biophys Res Commun* **401:** 598-604.

Shewry P. (2009). Wheat. *J Exp Bot* **60:** 1537-1553.

Song H., Tao Y., Ni N. N., Zhou X. W., Xiong J. S., Zeng X. S., Xu X. L., Qi J. L., Sun J. F. (2018). miR-128 targets the CC chemokine ligand 18 gene (CCL18) in cutaneous malignant melanoma progression. *Journal of Dermatological Science* **91:** 317-324.

Tao J., Liang W., An G., Zhang D. (2018). OsMADS6 Controls Flower Development by Activating Rice FACTOR OF DNA METHYLATION LIKE1. *Plant Physiol* **177:** 713-727.

Theissen G. (2001). Plant biology-floral quartets. *Nature* **409:** 469-471.

Thompson B., Bartling L., Whipple C., Hall D., Sakai H., Schmidt R., Hake S. (2009). bearded-ear encodes a MADS box transcription factor critical for maize floral development. *Plant Cell* **21:** 2578-2590.

Waddington S., Cartwright P., Wall P. (1983). A quantitative scale of spike initial and pistil development in barley and wheat. *Annual of Botany* **51:** 119–130.

Whipple C. J., Ciceri P., Padilla C. M., Ambrose B. A., Bandong S. L., Schmidt R. J. (2004). Conservation of B-class floral homeotic gene function between maize and Arabidopsis. *Development* **131:** 6083-6091.

Wu F., Shi X., Lin X., Liu Y., Chong K., Theissen G., Meng Z. (2017). The ABCs of flower development: mutational analysis of AP1/FUL-like genes in rice provides evidence for a homeotic (A)-function in grasses. *Plant Journal* **89:** 310-324.

Yadav S. R., Khanday I., Majhi B. B., Veluthambi K., Vijayraghavan U. (2011). Auxin-responsive OsMGH3, a common downstream target of OsMADS1 and OsMADS6, controls rice floret fertility. *Plant Cell Physiol* **52:** 2123-2135.

Yamaguchi T., Lee D., Miyao A., Hirochika H., An G., Hirano H. (2006). Functional diversification of the two C-class MADS box genes OSMADS3 and OSMADS58 in Oryza sativa. *Plant Cell* **18:** 15-28.

Yamaguchi T., Nagasawa N., Kawasaki S., Matsuoka M., Nagato Y., Hirano H. Y. (2004). The YABBY gene DROOPING LEAF regulates carpel specification and midrib development in Oryza sativa. *Plant Cell* **16:** 500-509.

Yoo S. K., Hong S. M., Lee J. S., Ahn J. H. (2011a). A genetic screen for leaf movement mutants identifies a potential role for AGAMOUS-LIKE 6 (AGL6) in circadian-clock control. *Mol Cells* **31:** 281-287.

Yoo S. K., Wu X. L., Lee J. S., Ahn J. H. (2011b). AGAMOUS-LIKE 6 is a floral promoter that negatively regulates the FLC/MAF clade genes and positively regulates FT in Arabidopsis. *Plant Journal* **65:** 62-76.

Zhang J. A., Nallamilli B. R., Mujahid H., Peng Z. H. (2010). OsMADS6 plays an essential role in endosperm nutrient accumulation and is subject to epigenetic regulation in rice (Oryza sativa). *Plant Journal* **64:** 604-617.

Zhang K., Liu J. X., Zhang Y., Yang Z. M., Gao C. X. (2015). Biolistic genetic transformation of a wide range of chinese elite wheat (Triticum aestivum L.) varieties. *Journal of Genetics and Genomics* **42:** 39-42.

Figures

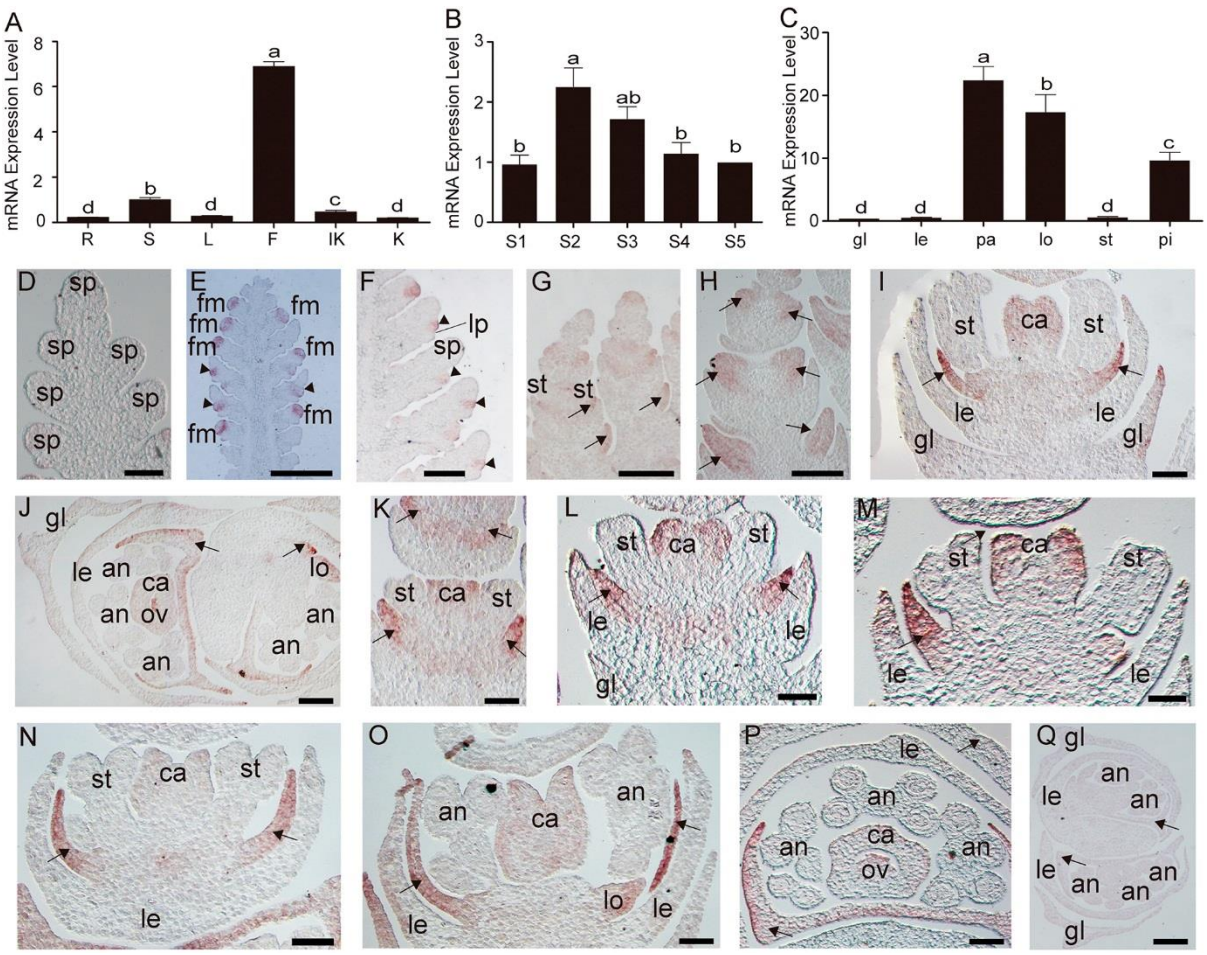


Fig.1 Expression profile of *TaAGL6* genes

(A) The expression in roots (R), stems (S), leaves (L), flowers (F), immature kernels (IK), and mature kernels (MK). (B) The expression in inflorescences or florets at different stages. S1 –S5, inflorescences at stages 3-3.25, 3.25-3.5, 4-5.5, 6-7 and 7.5-8.5, respectively. (C) The expression of *TaAGL6* genes in the glumes and floral organs at stage 6-7. Mean and SD values, and different letters in indicate significant differences ($P < 0.05$ by Student's *t*-test). (D–Q) Results of mRNA in situ hybridization. (D) No signal in one inflorescence at stage 2. (E) The transcripts in FMs at stage 3. (F) Signals in lodicule primordia at stage 3.5. (G and H)

The transcripts accumulated in the palea primordia, and paleas at stages 4. (I) Signals in the carpel primordium at stage 5. (J) The global expression pattern in florets at stage 6.5-7. (K–O) Wheat floret longitudinal sections to show *TaAGL6* mRNA signal accumulated in the paleas, lodicules, carpels, and ovules, whereas no obvious signal was detected in the stamens or anthers. K and L, stage 4.25 to 4.5; M and N, stage 5.5; O,stage 7. (P) One transverse section of a floret at stage 7.5. (Q) Negative control. A floret at stage 7 hybridized with sense probes. sp, spikelet; fm, FM; lp, lemma primordium; st, stamen; gl, glume; le, lemma; ca, carpel; lo, lodicule; ov, ovule; an, anther. Lodicule primordia are indicated by arrowheads, whereas paleas are indicated by arrows. Bars = 20 μm in F; 100 μm in D, G, K, and Q; 200 μm in H; 500 μm in E, I-P.

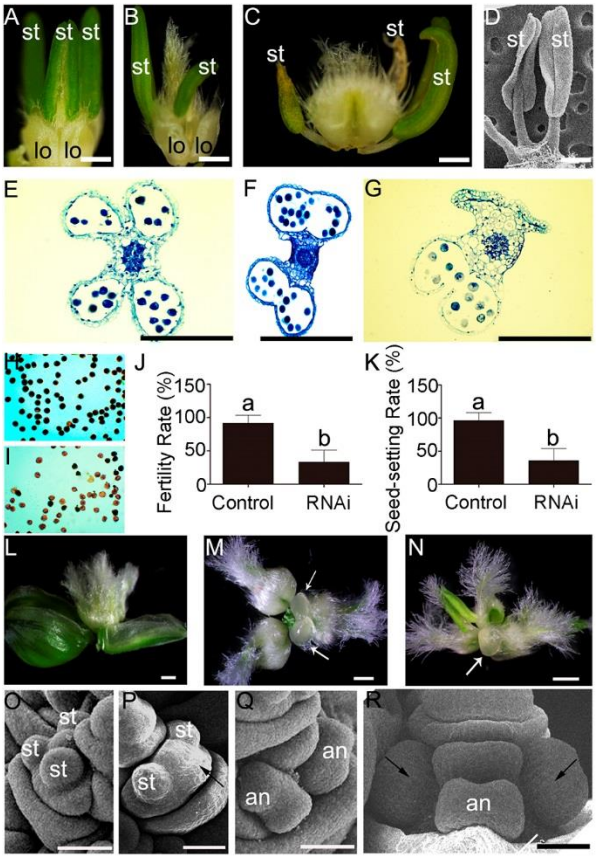


Fig.2 Phenotypes of *TaAGL6* RNAi stamens

(A) One wild type floret with three stamens. (B) One RNAi floret with a normal and abnormal stamen. (C) One RNAi floret with three abnormal stamens. The lemmas and paleas were removed in A–C. (D) SEM observation of abnormal RNAi stamens. (E–G) Histological analyses of wild type (E) and abnormal RNAi anthers (F and G). (H and I) Wild type (H) and RNAi pollen grains (I) stained with I_2-KI. (J) Fertility rate of pollen grains. (K) Seed-setting rate. (L–N) RNAi florets showing strong phenotypes (arrows indicate lodicules). The lemmas and paleas in M and N were removed. (O) SEM observation of one wild type floret with three stamen primordia. (P) SEM observation of one RNAi floret with two stamen primordia. (Q) SEM observation of one wild type floret to show the quadrangular stamens. (R) SEM observation of one RNAi floret to show two irregular stamens (indicated by black arrows). st, stamen; lo, lodicules. In J and K, mean and SD values in J and K were obtained from 30 replications (n = 30), and letters a and b in J and K indicate the significant differences according to Student's *t*-test ($P < 0.05$). Bars =100 μm in A to C, 20 μm in E to G, 50 μm in D and L to N, and 500 μm in O to R.

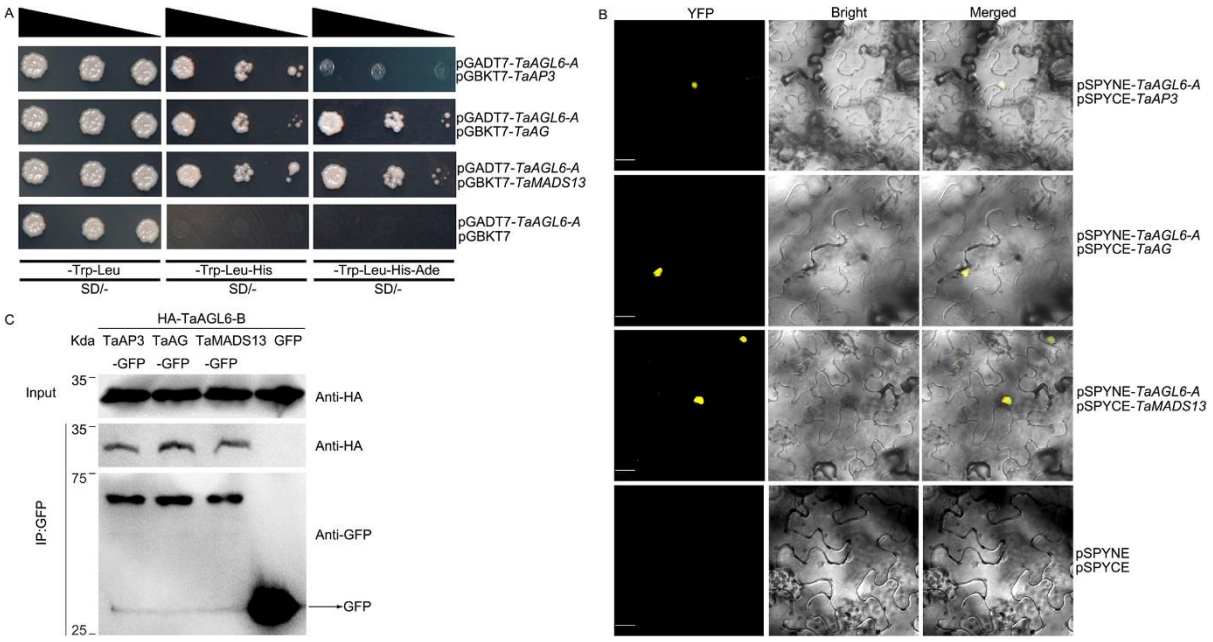


Fig. 3 Interactions between TaAGL6 and wheat floral regulators

(A) Results of Y2H to show interactions between TaAGL6 and TaAP3, TaAG, TaMADS13. (B) BiFC analyses to show the interactions in tobacco leaf cells (left, YFP; middle, Bright; right, Merged). (C) Results of Co-IP. The co-expressed HA-TaAGL6 and TaAP3-GFP, TaAG-GFP, TaMADS13-GFP, GFP proteins, were immuno-precipitated with GFP-Trap MA beads, and detected by GFP and HA antibodies respectively. Three replicates were performed for every experiment.

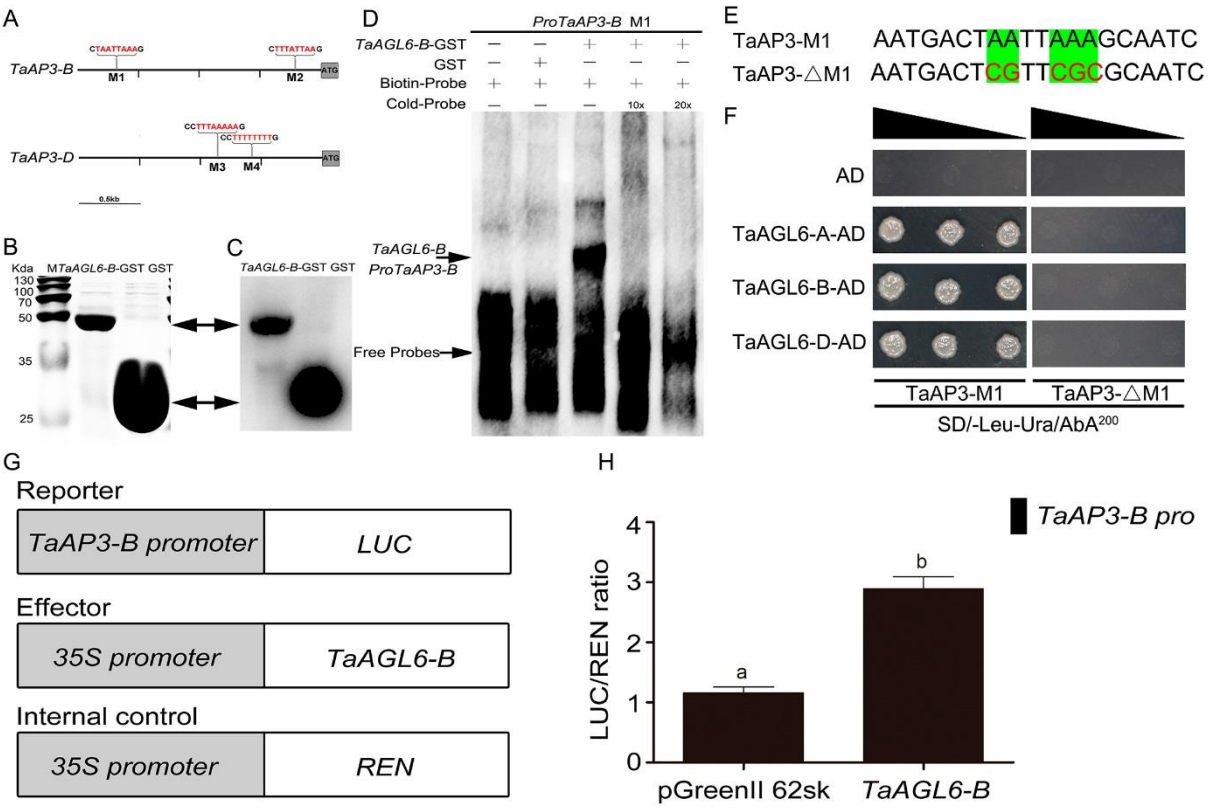


Fig. 4 Positive regulation of TaAGL6 Proteins to *TaAP3-B*

(A) CArG motifs in *TaAP3-B* and *TaAP3-D* promoters. (B) The purified TaAGL6-B-GST protein and GST. (C) Western blotting analysis of *TaAGL6*-B-GST protein and GST. (D) EMSA results. (E) M1 and mutated M1. The motif is indicated by green color, and the mutated bases are indicated by red color. (F) Y1H results showing the binding of TaAGL6 proteins to M1. pGADT7 vector is used as negative control. (G) Schematic of the reporter and effecter. (H) Relative reporter activity (LUC/REN) in tobacco leaves expressing the indicated reporter and effecter. Mean and SD values were obtained from three replications. Different letters indicate a significant difference ($P < 0.05$ by Student's *t* test).

Figure S1

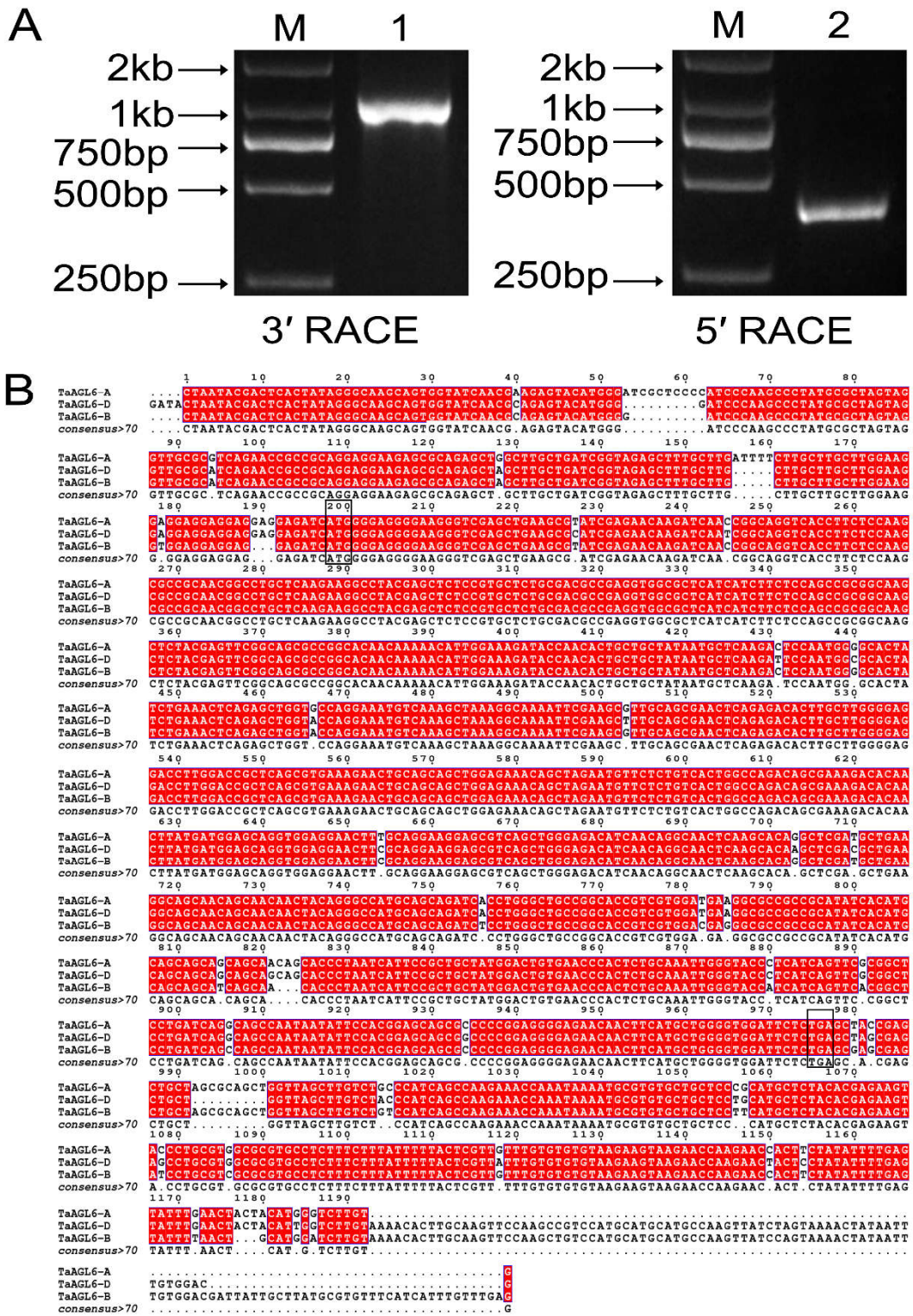
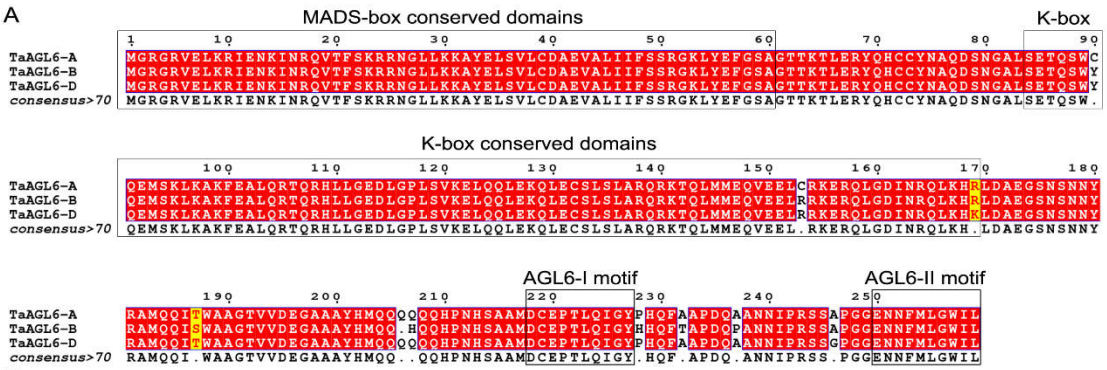


Fig. S1 Full cDNAs of *TaAGL6* genes.

(A) Results of RACE. (B) Alignment of *TaAGL6* full cDNAs. Rectangular boxes indicate the start and stop codons, respectively.

Figure S2

A



B

Variant	PROVEAN score	Prediction(cutoff=-2.5)
C90Y	6.431	Neutral
C153R	7.056	Neutral
Q206H	-0.219	Neutral
Q207del	0.379	Neutral
P228H	1.914	Neutral
A232T	-0.297	Neutral
A237P	-0.575	Neutral

C

Variant	PROVEAN score	Prediction(cutoff=-2.5)
C90Y	6.431	Neutral
C153R	7.056	Neutral
A246G	-0.842	Neutral

Fig. S2 Protein sequence and the effect of SNPs.

(A) Multiple allignment of TaAGL6 proteins. Rectangular boxes indicated MADS-box, K domains, AGL6 motifs I and II, respectively. (B and C) The effect of SNPs to the protein functions. Default threshold is -2.5, that is: The variant with a score equal to or below -2.5 is considered "deleterious," the variants with a score above -2.5 are considered "neutral."

Figure S3

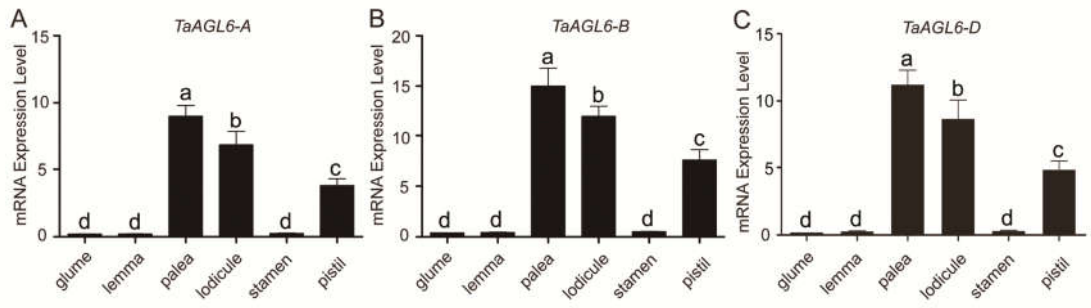


Fig. S3 The expression of *TaAGL6-A* (A), *TaAGL6-B* (B) and *TaAGL6-D* (C) in different floral organs.

Mean and SD values were obtained from three replications. Different letters indicate a significant difference ($P < 0.05$ by Student's *t* test).

Figure S4

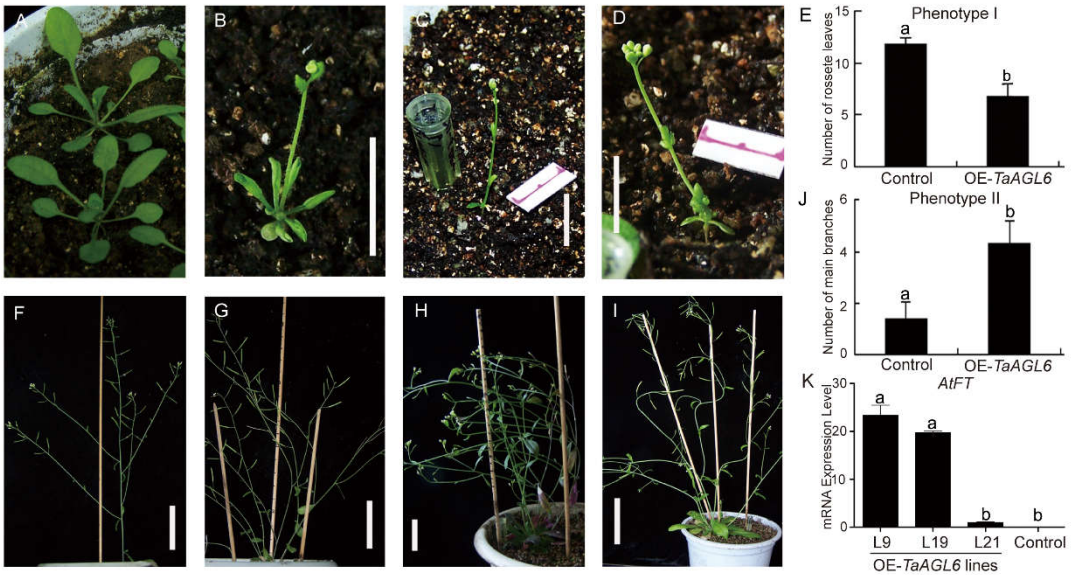


Fig.S4 Phenotypes of transgenic *Arabidopsis* over-expressing *TaAGL6* genes

(A-D) Four-week plants of wild type (A) and transgenic *Arabidopsis* overexpressing *TaAGL6-A* (B), *TaAGL6-B* (C), and *TaAGL6-D* (D) to show the early flowering phenotype of transgenic lines. (E) The number of rosette leaves in the control and in transgenic line overexpression *TaAGL6-B* at flowering time. (F-I) Wild type (F), transgenic *Arabidopsis* overexpressing *TaAGL6-A* (G), *TaAGL6-B* (H), and *TaAGL6-D* (I) to show the multi-shoot phenotypes. (J) The number of main stems in the control and transgenic *Arabidopsis* overexpressing *TaAGL6-B*. (K) The expression level of *AtFT* in different transgenic lines. Bars = 1cm in A to D, F to I. In E and J, mean and SD values were obtained from 30 replications (n=30), and letters a and b indicated the significant difference between the control and transgenic plants according to Student's *t*-test (P<0.05). Mean and SD values in K were obtained from three replications, and different letters in K indicated a significant difference ($P < 0.05$ by Student's *t* test).

Figure S5

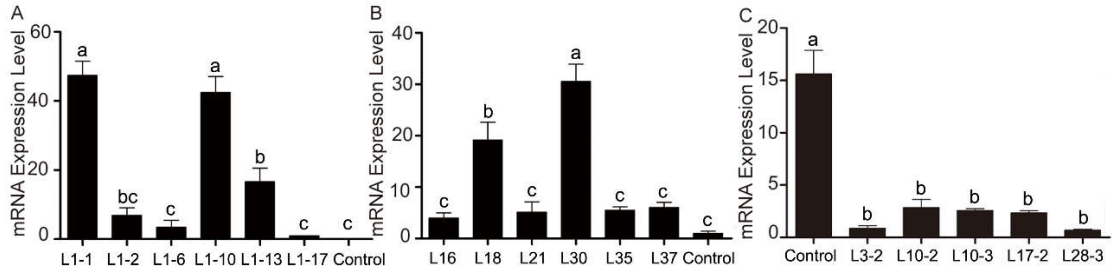


Fig. S5 The expression of *TaAGL6* genes in transgenic *Arabidopsis* (A), transgenic wheat (B) overexpressing *TaAGL6-B*, and *TaAGL6* RNAi wheat (C).

Mean and SD values were obtained from three replications, and different letters indicated significant differences ($P < 0.05$ by Student's *t* test).

Figure S6

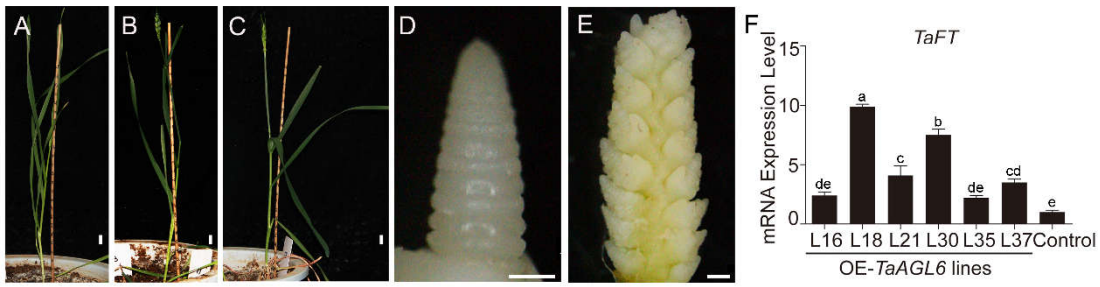


Fig. S6 Phenotype of transgenic wheat over-expressing *TaAGL6-B*.

(A-C) One plant of control (A) and transgenic Line 11 (B) and Line 18 (C) photographed at the same time. (D and E) One inflorescence of control (D) and one inforescence of transgenic plants (E) collected at the same time. (F) The expression of *TaFT* in different transgenic lines at vegetative stage. Bars = 1cm in A-C, 50μm in D and E. Mean and SD values in F were obtained from three replications, and different letters in F indicated a significant difference ($P < 0.05$ by Student's *t* test).

Figure S7

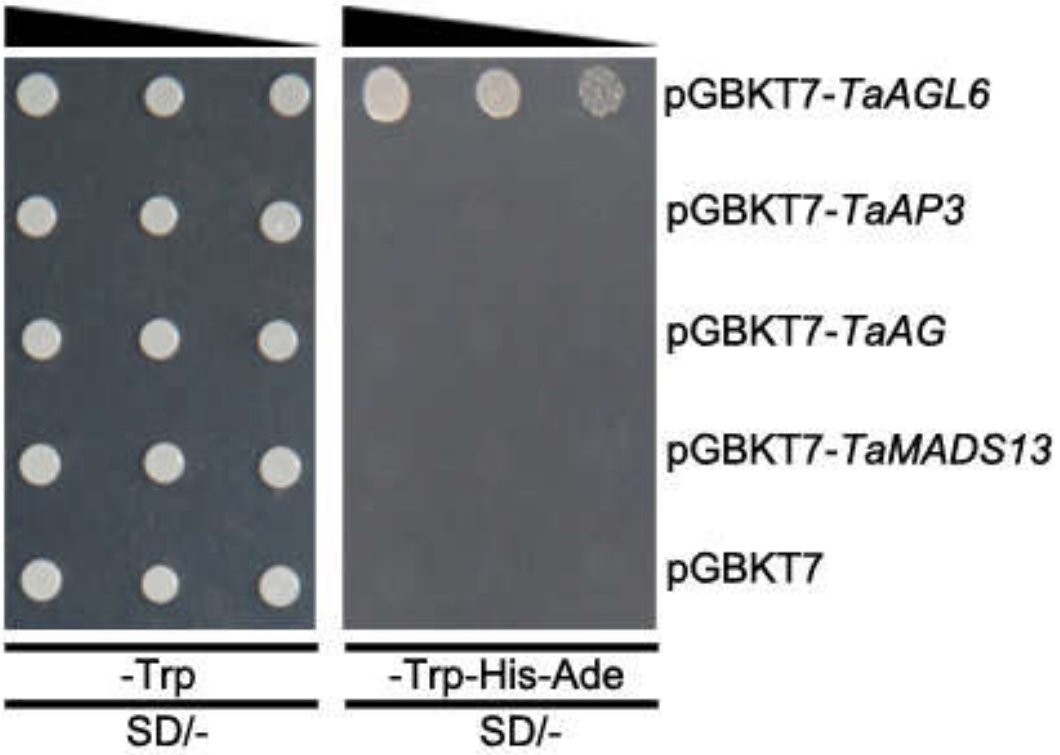


Fig. S7 Self-activation assays of TaAGL6, TaAP3, TaAG, and TaMADS13.

Figure S8

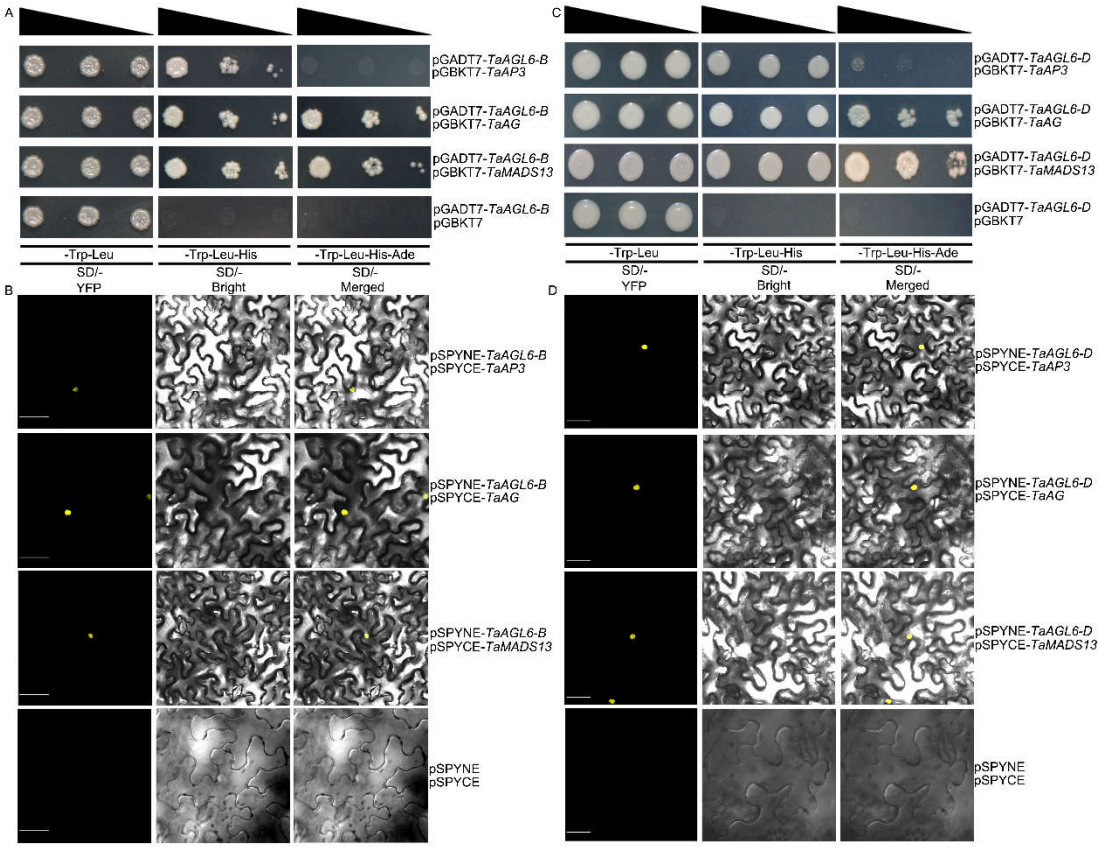


Fig. S8 Interactions between TaAGL6-B/TaAGL6-D and TaAP3, TaAG, TaMADS13. (A and B) Interactions between TaAGL6-B and TaAP3, TaAG, TaMADS13 in yeast cells (A), and tobacco leaf cells (B). (C and D) Interactions between TaAGL6-D and TaAP3, TaAG, TaMADS13 in yeast cells (C), and tobacco leaf cells (D). In B and D, left, YFP; middle, Bright; right, Merged.

Figure S9

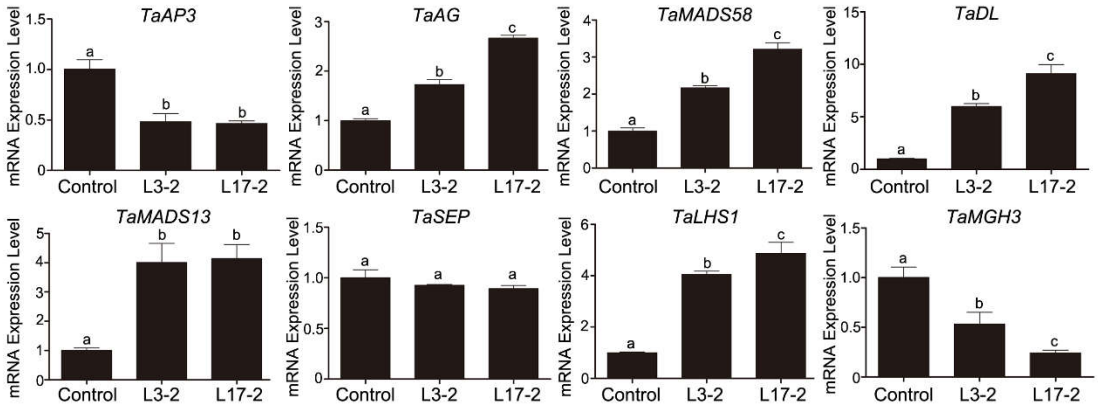


Fig. S9 The expression of wheat floral genes and *TaMGH3* in wild type and *TaAGL6* RNAi stamens.

Mean and SD values in were obtained from three replications, and different letters indicated significant differences ($P < 0.05$ by Student's *t* test).

Figure S10

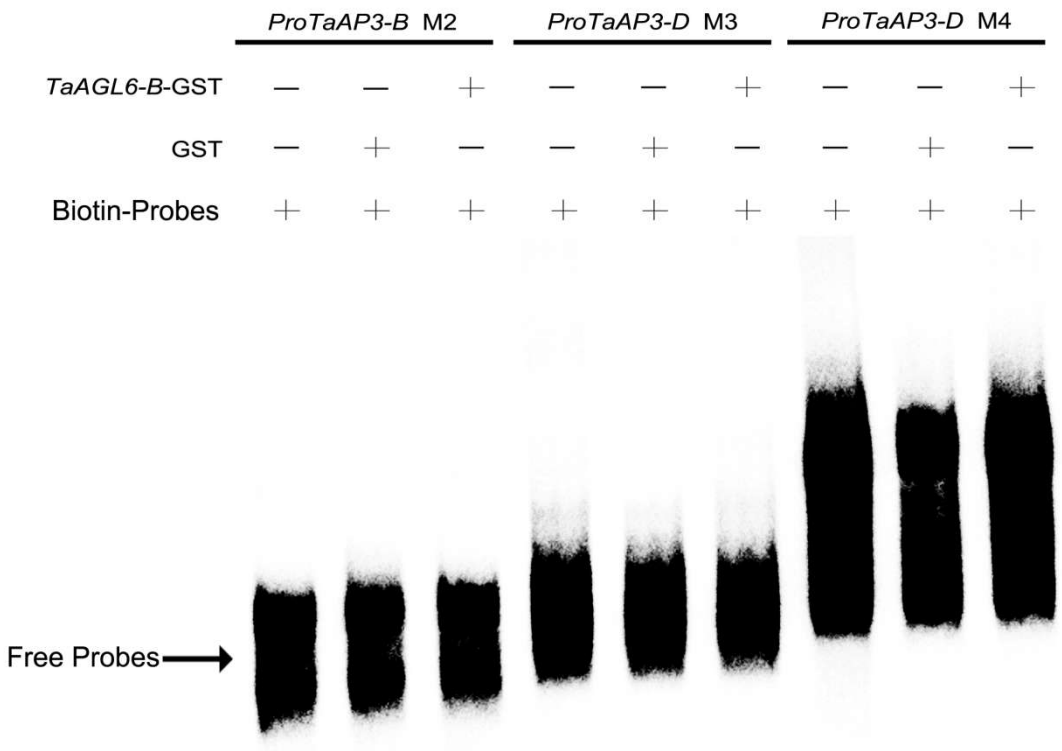


Fig. S10 Results of EMSA to show that AGL6 proteins could not bind to CArG motifs 2-4.

Figure S11

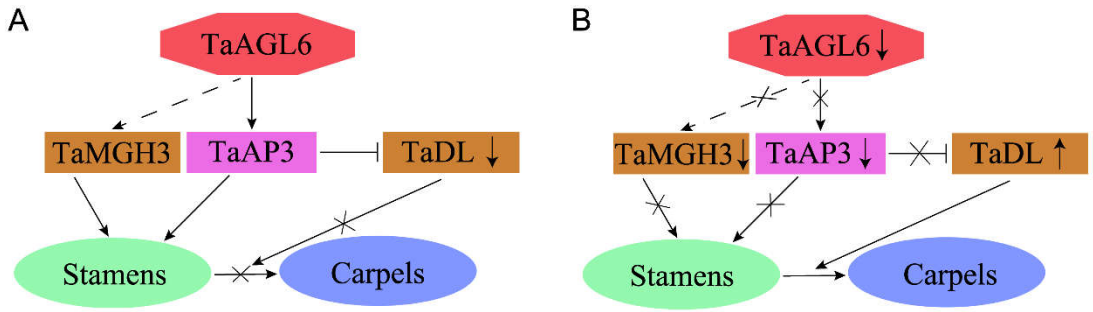


Fig. S11 Proposed model to illustrate the function and mechanism of TaAGL6 in stamen development

(A) In wild type wheat, TaAGL6 transcription factors directly and indirectly regulate the expression of *TaAP3* and *TaMGH3*, respectively. *TaAP3* and *TaMGH3* function in stamen development. Meanwhile, *TaAP3* represses the expression of *TaDL* in stamens. As a result, stamens develop normally. (B) In *TaAGL6* RNAi plants, the expression of *TaAGL6* genes is down-regulated. Consequently, the expression of *TaAP3* and *TaMGH3* is down-regulated, while *TaDL* is ectopically expressed in stamens. As a result, the stamens develop abnormally, and display the potential to transform into carpels.

Table S1. Primers used in this research.

Name	Sequence（5’-3’）
3GSP	ATGGGGAGGGGAAGGGTCGAG
5GSP	ATCTTTCCAATGTTTTTGTTGTGCC
TaAGL6ADLF	TAGTAGGTTGCGCGTCAGAA
TaAGL6ADLR	CCTCCTTCCAAGCAAGCAAGAAAAT
TaAGL6BDLF	GAAGGCAGCAACAGCAACAA
TaAGL6BDLR	GGAATGATTAGGGTGTTGCTGA
TaAGL6DDLF	GTACCCTCATCAGTTCGCGG
TaAGL6DDLR	ACAAGCTAACCAGCAGCTCGCT
TaAGL6DLF	CTTGCTTGGGGAGGACCTTGGA
TaAGL6DLR	CATCATAAGTTGTGTCTTTCGCTGT
TaAGL6RNAiPF	GGTGGTAAGCTTGCGGCCGCTGAAGGCAGCAACAGCAACAACTA
TaAGL6RNAiPR	GGTGGTGAATTCGGATCCATGGACAGCTTGGAACTTGCA
T7 promoter sequence	TAATACGACTCACTATAGGG
TaAGL6T7-F	TAATACGACTCACTATAGGGCTGAAGGCAGCAACAGCAACAACTA
TaAGL6T7-R	TAATACGACTCACTATAGGGATGGACAGCTTGGAACTTGCAA
TaActinPF	TATGCCAGCGGTCGAACAAC
TaActinPR	GGAACAGCACCTCAGGGCAC
TaAGL6OF	GGTGGTCCATGGGGAGGGGAAGGGTCGAG
TaAGL6OR	GGTGGTCACGTGTCAGAGAATCCACCCCAGCAT
1301PF	GTGATATCTCCACTGACGTAAGGG
1301PR	GATAATCATCGCAAGACCGGCA
pBSKR	GACAGCAGCAGTTTCATCAATCA
AtGAPCPF	TCAGACTCGAGAAAGCTGCTACC
AtGAPCPR	GATCAAGTCGACCACACGGGAA
TaAGL6PF	ATGGGGAGGGGAAGGGTC

TaAGL6PR	TCAGAGAATCCACCCCAGCAT
TaAP3PF	ATGGGGCGGGGGAAGAT
TaAP3PR	TTAGCCGAGGCGCAGGTC
TaAGPF	ATGCAGATACTCAACGAGCAGCT
TaAGPR	TCACCTTCCAACTGAGTT
TaMADS13PF	ATGGGGAGGGGAAGGATTG
TaMADS13PR	CTAGAACTGATGAGCCACATCGC
TaAP3DLF	AGGAGGCATACAAGAATCTGCA
TaAP3DLR	GCTAGTAGGAGCGATCGAAGTGA
TaAGDLF	TACTCCAACAACAGCGTGAAAGC
TaAGDLR	GTATCGCCTATTAGAGTCCTGTTGG
TaMADS58DLF	ATCAAGCGCATCGAGAACAC
TaMADS58DLR	ATGGTTGCTTTCACGCTGTT
TaDLDLF	AACCTCTCCTTTCTCAGCCC
TaDLDLR	GGGCTTCACAACAAAGGGAG
TaMADS13DLF	TCAGAACCAAGATTGCGGAGGA
TaMADS13DLR	CTAGAACTGATGAGCCACATCGC
TaSEPDLF	AAGAAGGCCTACGAGCTCTC
TaSEPDLR	GGTACTCATTGCGGCTGTTT
TaLHS1DLF	CTCAAGCATATCAGGTCAAAAAAGAATCAA
TaLHS1DLR	TCAGAAGCCACGTGATCTCTGTT
TaMGH3DLF	CCTACATCCAGCGCATTGTC
TaMGH3DLR	ACGAACAGGAAGTAGAGGCC
TaAP3BCARG1PROBEF	AAAAGATCTTTTCGTTCCAGAAGAA
TaAP3BCARG1PROBER	GGTAGCCAAAAAATTCTAAATACCA
TaAP3BCARG2PROBEF	TGCCCGTTCTATTCT
TaAP3BCARG2PROBER	ATCATTGCTTCGCTGCTTT
TaAP3DCARG1PROBEF	GAACGCTAGCTAAGCCATAGG
TaAP3DCARG1PROBER	CTGTCCACTTCCAAAAGAGGT

TaAP3DCARG2PROBEF	CCTTCTTCCTCCTCCTA
TaAP3DCARG2PROBER	TGGATAGAAGGGGCATTGTCT
TaAGL6-BGSTF	CCTGGGATCCCCGGAATTCATGGGGAGGGGAAGGGTC
TaAGL6-BGSTR	GTCACGATGCGGCCGCTCGAGTCACCTGTGCTTGAGTTGCCTGTT
TaAP3F	AAGCTTGAATTCGAGCTC
	GACTAATTAAAGCAGACTAATTAAAGCAGACTAATTAAAGCA
	GTCGACCTCGAGGCATGT
TaAP3R	ACATGCCTCGAGGTCGAC
	TGCTTTAATTAGTCTGCTTTAATTAGTCTGCTTTAATTAGTC
	GAGCTCGAATTCAAGCTT
Mut TaAP3F	AAGCTTGAATTCGAGCTC
	GACTCGTTCGCGCAGACTCGTTCGCGCAGACTCGTTCGCGCA
	GTCGACCTCGAGGCATGT
Mut TaAP3R	ACATGCCTCGAGGTCGAC
	TGCGCGAACGAGTCTGCGCGAACGAGTCTGCGCGAACGAGTC
	GAGCTCGAATTCAAGCTT
TaAGL6-BOE6HAF	GTCGACGGTATCGAT AAGCTT ATGGGGAGGGGAAGGGTCGAG
TaAGL6-BOE6HAR	AGAACTAGTGGATCC CCCGGG GAGAATCCACCCCAGCAT
TaAP31302GFPF	CATGGTAGATCTG ACTAGT ATGGGGCGGGGGAAGAT
TaAP31302GFPR	GCCCTTGCTCACCAT CCTAGG GCCGAGGCGCAGGTC
TaAG 1302GFPF	CATGGTAGATCTG ACTAGT ATGCAGATACTCAACGAGCAGCT
TaAG 1302GFPR	GCCCTTGCTCACCAT CCTAGG CCTTCCAACTGAGTT
TaMADS13GFPF	CATGGTAGATCTG ACTAGT ATGGGGAGGGGAAGGATTG
TaMADS13GFPR	GCCCTTGCTCACCAT CCTAGG GAACTGATGAGCCACATCGC
TaAGL6-B 62SKF	CGCTCTAGAACTAGT GGATCC ATGGGGAGGGGAAGGGTCGAG
TaAGL6-B 62SKR	GTCGACGGTATCGAT AAGCTT TCAGAGAATCCACCCCAGCAT
TaAP3B-0800LUCF	GGCCCCCCCTCGAG GTCGACAAAAGATCTTTTCGTTCCAGAAGAA
TaAP3B-0800LUCR	GCTCTAGAACTAGT GGATCC GGGGCGGCCGTGGTTTTGA