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Overexpression of *AGAMOUS-like* gene *PfAG5* promote early flowering in *Polypogon fugax*

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Abstract

Background: Herbicides are the major tool for controlling large populations of yield depleting weeds. However, overreliance on herbicides has resulted in weed adaptation and herbicide resistance. In recent years, early flowering weed species related to herbicide resistance is emerging, which may cause seed loss before crop harvest, creating a new problem for non-chemical weed management. However, mechanisms regulating early flowering in weedy species is rarely investigated.

Results: The MADS-box gene family plays an important role in flowering time regulation and floral organogenesis. In this study, a homolog gene of *AGAMOUS* sub-family (referred to as *PfAG5*) of the MADS-box family was cloned from plants of an early flowering *Polypogon fugax* population resistant to the ACCase inhibitor herbicide (clodinafop-propargyl). The *PfAG5* gene was functionally characterized in *Arabidopsis thaliana*. Over-expression of the *PfAG5* gene in Arabidopsis resulted in early flowering with abnormal flowers (e.g. small petals, short plants and reduced seed set) compared to the wild type. The expression of the *PfAG5* gene was high in leaves and flowers, but low in pods in transgenic Arabidopsis. The *PfAG5* gene was earlier and higher expressed in the resistant (R) than the susceptible (S) *P. fugax* plants. Furthermore, one protein (*FRIGIDA*-like protein) interacting with *PfAG5* in R *P. fugax* was identified by the yeast two-hybrid system with relevance to flowering time regulation.

Conclusions: These results suggest that the *PfAG5* gene is prominently involved in modulating early flowering in *P. fugax*. This study provides the first evidence for the regulation mechanism of early flowering in an herbicide resistant weed species.

Background

Flowering is the most dramatic transition from the vegetative phase to reproductive phase in a life cycle of flowering plants, and hence an important agronomic trait. To achieve reproductive success, the optimal flower timing is critical for flowering plants [1]. Flowering time is regulated autonomously and by environmental factors, such as stress-induced flowering in addition to photoperiodic flowering and vernalization [2].

Many stress factors have been reported to induce flowering, such as light intensity, UV light, temperature, nutrition and drought, as summarized in previous reviews [3–4]. Herbicide application is also a stress to weedy plants, and it can select for evolution of herbicide resistance [5]. Adaptive changes in seed germination and seedling emergence, flower bud formation and flowering time have also been observed in herbicide resistant biotypes [6–10].

In agriculture, frequent and regular disturbances from plowing and harvesting likely exert a strong selection on weeds for rapid flowering and seed set [11]. For example, highly effective weed seed collection techniques at harvest may exert intense selection for earlier flowering (likely early seed shattering) phenotypes to evade collection, and genetically diverse *Raphanus raphanistrum* exhibited significant standing genetic variations to adapt to flowering time selection [12]. The ability to reach

inflorescence emergence and flowering earlier is an advantage to weed populations allowing them to escape potential eradication by late-season weed management strategies or harvesting [13].

MADS-box genes are key regulators of many aspects of plant reproductive development, especially in flowering time control, inflorescence architecture, floral organ identity determination and seed development. Based on their evolutionary origin, MADS-box genes have been divided into two classes, namely, type I and II. The plant-specific type II MIKC MADS box genes are key regulators of developmental processes, such as flowering time, fruit and seed development [14]. In Arabidopsis, four genes, *AGAMOUS* (*AG*), *SHATTER PROOF1* (*SHP1*), *SHP2* and *SEEDSTICK* (*STK*), compose the monophyletic *AG*-subfamily within the MADS-box gene phylogeny [15–16]. Members of the *AG*-subfamily are involved in the specification of floral reproductive organs and required for normal development of carpels and fruits [17]. For instance, when expressed in Arabidopsis the *AG*-subfamily genes from *Gossypium hirsutum* regulate flower development and fruit formation [18]. The MIKC-type genes can be subdivided into 12 major gene clades, including floral promoters (e.g. *AGAMOUS-like24*, *SOC1*) and repressors (e.g. *FLM/MAF1*, *FLC*). These flowering genes generally exert their function by influencing 'meristem identity genes' which control the transition from inflorescence to floral meristems [19].

In our previous study, we found that the R *Polypogon fugax* population was resistant to ACCase-inhibiting herbicides clodinafop-propargyl, fluazifop-p-butyl, haloxyfop-R-methyl, quizalofop-p-ethyl and fenoxaprop-p-ethyl, relative to the susceptible population [20]. Plants of the R *P. fugax* population were found earlier in head emergence, flowering, and seed maturation than that of the S population [21]. Transcriptome analysis identified a flowering-related contig (CL10710.contig2) belonging to the *AGAMOUS*-subfamily of the MADS-box gene family, that had significantly higher expression at the flowering stage in the R *P. fugax* versus the S *P. fugax* [22]. To determine the role of the contig (CL10710.contig2, and thereafter named as *PfAG5*) in flowering time regulation, we cloned the full-length cDNA sequence of the *PfAG5* gene from R *P. fugax* and transformed Arabidopsis. We analyzed the expression pattern of *PfAG5* gene as well as other six endogenous flowering regulation genes in transgenic Arabidopsis plants. Furthermore, we identified a *PfAG5* interaction protein in R *P. fugax* by the yeast two-hybrid system. This is the first study on the regulation mechanism of early flowering in a weedy plant species. This knowledge will aid in future genetic approaches for better weed control strategies.

Results

Cloning of PfAG5 **cDNA coding sequence from R** P. fugax

The *PfAG5* coding sequence (GenBank accession number MK559453) is 831 bp encoding a 277-amino acid protein with 91% and 88% identity respectively to *Hordeum vulgare* subsp. Vulgare *AGAMOUS*-like protein 1 *HvAG1*(AAL93196.1) and *Aegilops tauschii WAG-2f* MADS box transcription factor (ALM58837.1). A phylogenetic tree was constructed to determine the relationship of PfAG5 protein with AGAMOUS-like proteins of other plant species. As shown in Supplementary Fig. S1A, PfAG5 belongs to the family of AGAMOUS homologs from monocots, and is closely related to AGAMOUS-like proteins from

H. vulgare subsp. Vulgare, *Triticum aestivum* and *A. tauschii*. Sequence alignment revealed that *PfAG5* has a conserved DNA-binding SRF-type TF domain, MADS-box domain and AG Motif (Supplementary Fig. S1B).

Overexpression of PfAG5 in Arabidopsis induces early flowering with abnormal flowers

Phenotypes of 20 independent homozygous T3 transgenic lines were examined. Arabidopsis plants overexpressing *PfAG5* flowered 15–16 days earlier and produced 5–6 fewer rosette leaves than wild type Arabidopsis (WT) and empty plasmid transgenic (Mock) plants under long day (LD) conditions (Table 1, Fig. 1A). Under short day (SD) conditions, *PfAG5* transgenic plants flowered approximately 50–60 days earlier and produced 35–38 fewer rosette leaves than control plants (Table 1, Fig. 1B).

Table 1
Changes in growth and reproduction of *Arabidopsis thaliana* overexpressing the *PfAG5* gene under long day or short day conditions. Data are mean ± SE (n = 20 lines)

Treatment	Rosette leaves	Flower time(day)	Pod Numbers	Plant Height (cm)	Seed Yields (g plant^{-1})
Long day condition					
WT	14.0 ± 0.25 ^{b}	27.7 ± 0.23 ^{b}	272 ± 12 ^{b}	38.2 ± 0.48 ^{b}	0.1466 ± 0.0023 ^{b}
PfAG5	9.0 ± 0.22 ^{a}	12.9 ± 0.17 ^{a}	43 ± 4^{a}	12.9 ± 0.93 ^{a}	0.0113 ± 0.0013 ^{a}
Mock	14.0 ± 0.27 ^{b}	28.5 ± 0.23 ^{b}	276 ± 9^{b}	41.4 ± 1.19 ^{b}	0.1461 ± 0.0052 ^{b}
Short day condition					
WT	47.0 ± 1.12 ^{b}	77.8 ± 0.95 ^{b}	63 ± 1^{b}	49.0 ± 0.36 ^{b}	0.0323 ± 0.0007 ^{b}
PfAG5	10.5 ± 0.17^{a}	21.5 ± 0.31 ^{a}	15 ± 1^{a}	20.1 ± 0.28 ^{a}	0.0033 ± 0.0001 ^{a}
Mock	45.1 ± 0.90 ^{b}	81.2 ± 1.60 ^{b}	64 ± 1^{b}	50.3 ± 0.31 ^{b}	0.0326 ± 0.0006 ^{b}
Different letters in a column indicate significant difference by Ducan's multiple comparison test $P < 0.01$					

In contrast to control plants, all the *PfAG5* transgenic Arabidopsis plants displayed abnormal growth as narrow and curly leaves in the seedling stage, and very short petals (Fig. 2). No differences were observed in morphology of pods and seeds (Supplementary Fig. S2), but plant height, pod number and seed yield were all lower in *PfAG5* transgenic plants than in WT and Mock controls (Fig. 1 and Table 1). Thus, expression of *PfAG5* in Arabidopsis resulted in the phenotype of early-flowering with abnormal flowers.

Expression pattern of PfAG5 and Endogenous Genes Involved in Flowering in transgenic Arabidopsis

Expression pattern of *pfAG5* in different tissues of transgenic Arabidopsis plants (*35S::PfAG5*) were analyzed by qRT-PCR. Results showed that *PfAG5* was constitutively expressed in leaves and flowers, and

the expression level of *PfAG5* was significantly higher in leaves and flowers, but lower in young pods than controls (Fig. 3). This is similar to the MADS-box gene of *Brachypodium distachyon BdMADS33* which showed weak expression signal in young seed [23].

In *PfAG5* transgenic Arabidopsis plants, higher expression of other Arabidopsis endogenous genes CONSTANS (*CO*), SUPPRESSOR OF CONSTANS OVEREXPRESSION1 (*SOC1*), and lower expression of the FLOWERING LOCUS C (*FLC*) gene were found in comparison to WT, in the whole-above ground material at the flowering stage. No difference in APETALA1 (*AP1*) expression was found (Fig. 4).

Identification of PfAG5 **interaction proteins in R** P. fugax

Three proteins (AD1, AD2, AD3) interacting with *PfAG5* in R *P. fugax* were identified by the yeast two-hybrid system and validated as positives in yeast by one-to-one interaction experiment (Fig. 5A).

The AD1 showing 91% and 87% amino acid sequence identity to *A. tauschii* subsp. tauschii *FRIGIDA-like* protein 3 (XM_020321692) and *B. distachyon FRIGIDA- like* protein 3 (XM_003577031.4), respectively. The AD2 showing 82% and 77% sequence identity to *A. tauschii* subsp. tauschii *Agamous*-like MADS-box protein *AGL66* (XM_020339220.1) and *Panicum hallii Agamous*-like MADS-box protein *AGL104* (XM_025966163.1), respectively. And the AD3 showing 93% sequence identity to *Lolium perenne* MADS2 (AY198327.1) and *Avena sativa* fruitful-like MADS-box transcription factor (*FUL2*) (DQ792967.1), respectively.

Expression pattern of PfAG5 **and the interacting proteins in** P. fugax

The expression patterns of *PfAG5* and the three interacting proteins were compared at different developmental stages (the seedling, tillering and flowering stages) and between R and S plants. The early flowering stage of the R plants corresponded to the heading stage of the S plants. The expression of *PfAG5* in both S and R plants was significantly increased (by 5.7- and 10.2-fold, respectively) at the flowering stage as compared to the seedling stage. However, *PfAG5* expression was significantly higher in the R than the S plants at the tillering and flowering stages (Fig. 5B). For, instance, the transcript level of *PfAG5* reached 3-fold higher in the early flowering stage of R than that of S (while S still at the heading stage) (Fig. 5B).

Conversely, the expression of AD-1 was 1.86-fold lower at the early flowering stage of R than the S plants, while there was no significant differences at the seedling and tillering stages. While the expression of AD-2 was consistently lower in the R than in the S plants at all stages, there was no clear pattern in the expression of AD-3 (Fig. 5B).

Discussion

Flowering time of many weedy species is synchronized with that of crops [24], so weeds often mature concurrently with crops. Due to herbicide and non-herbicide weed control selection pressures, changes in weed growth and reproduction have been evolved to adapt to the environment, including changes in

flowering time [6, 12]. For instance, in a glyphosate resistant population of *Conyza bonariensis* from Brazil, the first floral bud formation was observed 28 days earlier than the glyphosate susceptible population [9]. An ALS resistant population of *Apera spica-venti* flowered 13 days earlier than the susceptible population at a certain crop density [10]. Panicles of the ACCase herbicide resistant (due to the 2041 mutation) *Hordeum glaucum* biotype emerged approximately 20 days earlier than that of susceptible biotype in the field [25]. The ACCase herbicide resistant (due to the 1781 mutation) *Setaria viridis* plants flowered and matured earlier, but producing 24% more seeds than the susceptible plants [6].

In our previous study, we found that an ACCase inhibitor resistant (due to the 2041 mutation) *P. fugax* population reached the tiller and panicle emergence and seed shedding stages 6, 10 and 12 days respectively earlier than the S population [26]. Working with this early flowering population, we identify an *AGAMOUS*- subfamily gene *PfAG5* that is involved in early flowering in R population in this study. The AG-like gene subfamily includes members involved in the specification of stamen, carpel and ovule. Phylogenetic analysis showed that *PfAG5* groups into AG-like clade in MADS-box genes of other plants, and is homologous to the *A. tauschii* AG-type genes as *WAG-2f* and *WAG-2 g* [27], and *Triticum aestivum TaAGL39* [28]. Overexpression of *AGL79* in Arabidopsis result in the narrow leaf shape, fewer number of leaves and early flowering [29], which are consistent with the phenotypes of *PfAG5* transgenic Arabidopsis plants.

It is known in Arabidopsis that the floral integrator FLOWERING LOCUS T (*FT*) is a key regulator of flowering time [30], and transcription factor *CO* activates the expression of *FT* [31], promoting early flowering. In deed in our experiment, we found that the expression of *FT* and *CO* in transgenic Arabidopsis (*35S::PfAG5*) were significantly higher than in WT (Fig. 4). So, we speculate that the *PfAG5* gene promotes the expression of *CO* in transgenic plants, and the high expression of *CO* in turn activates the expression of *FT*. In contrast, the expression of *FLC* can represses the transcriptional activation of the floral integrator genes *FT* and *SOC1* [32], hence inhibiting flowering. In this study, *FLC* expression was inhibited in *PfAG5* transgenic plants, which may release repression of *FT* and *SOC1* (Fig. 4).

Available genetic and molecular evidence suggests that *LFY* and *AP1* together orchestrate the switch to flower formation and early events during flower morphogenesis by altering transcriptional programs [33]. It is known that *AP1* plays a role in differentiation of sepals and petals [34]. However, in the current study, no difference in the expression of *AP1* was detected in *PfAG5* transgenic Arabidopsis plants and WT at the flowering stage. In this case we speculate that the morphological change in petals of *PfAG5* transgenic plants may be related to genes other than AP1. Similarly, expression of *DcaAP1*, *DcaAP2* and *DcaAP3* in *Dianthus caryphyllus* did not significantly differ in petals of different flower phenotypes [35]. It was found that overexpression of LEAFY (*LFY*) resulted in early flowering [36], likely via causing precocious development of flowers, converting the inflorescence shoot into a single terminal flower [37]. So the high expression of *LFY* in *PfAG5* transgenic plants may be related to the early flowering and abnormal flower (Fig. 4).

Plants with a shorter vegetative phase have less time to build up resource-gathering organs for seed production, so early flowering can be expected to decrease the reproductive output [38]. Indeed, we found that overexpression of *PfAG5* in Arabidopsis resulted in not only early flowering and flower morphological changes, but also significant decline in seed production (Table 1). Arabidopsis plants transformed with Carnation AG genes (*DcaAGa*, *DcaAGb*) also showed petal loss, short silique, and seed sterility [35], and this is similar to the flower phenotype of *PfAG5* transgenic plants, except for seed activity. These results imply that *PfAG5* gene is a flowering time promoter for the efficient expression of other flowering time regulatory genes, causing early flowering and abnormal flowers.

But how about the possible flowering regulation pathways of *PfAG5* in R *P. fugax* population? In this current study we identified three *PfAG5* interacting proteins sequences of AD-1, AD-2 and AD-3, with homology to *FRI3*, *AGL66* and *FUL2* gene respectively. In Arabidopsis, the *FRI* promotes later flowering by enhancing the expression of the flowering repressor gene *FLC* [39], and RNA silencing of *FRI*-like protein 3 (*FRL3*) mRNA induces early flowering in tomato plants [40]. Despite the central role of *FLC*, most of the variations in flowering time have been correlated with natural allelic diversity of *FRI* [41]. For instance, among *FRIGIDA* orthologues the *BnaA3.FRI* is tightly associated with flowering time variation in *B. napus* [42]. In our study, the *FRI-like* gene (*AD-1*) was inhibited at the flowering stage of R *P. fugax* plants, contrary to the high expression of *PfAG5* (Fig. 5B). Therefore, inhibition of the *FRI* (AD-1) gene caused by overexpression of the *PfAG5* gene is likely responsible for early flowering in the resistant *P. fugax* R population.

AGAMOUS LIKE-66 (*AGL66*), together with other *AGL* genes, constituted a regulatory network for regulating the germination of male germ cells which is related to the flower formation [43]. In this study, the expression of the homology gene *AD-2* was found to be consistently lower in the R than in S *P. fugax* populations (Fig. 5B). We speculate that the inflorescence morphological changes in *P. fugax* R population [26] may be related to the expression of this gene. As there was no clear trend in the expression of *AD-3* (homology to *FUL*), and as *AP1/ FUL* gene (*FUL2*) may play a general role in regulating flowering time in monocots [44], we assume that *AD-3* may not play a major role in flowering regulation in *P. fugax*.

Flowering time regulation is a coherent and sophisticated event, involving many genes. But how herbicide resistant (R) *P. fugax* have got higher expression of the flowering genes such as PfAG5 as compared to herbicide susceptible (S) population? Or how early flowering trait is correlated to herbicide resistance? According to Baucam [45], an alteration in a life-history trait in a resistant lineage can be caused by the resistance allele itself (a pleiotropic effect) or could result from genetic linkage between the resistance allele and genes that control the life-history trait. However, the herbicide resistance allele in the R *P. fugax* population was a point Ile-2041-Asn mutation in the plastidic ACCase gene [46], and there has no evidence showing ACCase correlation with flowering time regulation. Rather, genetic linkage between the resistance ACCase allele and flowering genes may be possible. Standing genetic variations in flowering time may exist in *P. fugax* populations, herbicide application may not only have selected for herbicide resistance but also by chance for plants with higher expression of flowering genes. Alternatively, higher

expression of flowering genes can be induced by herbicide application, and becomes fixed overtime by such as epigenetic mechanisms in plants having the herbicide resistance allele. The letter can be examined by methylation analysis of the candidate flowering genes. Nevertheless, early flowering (likely early pod shedding) will be a disadvantage for later season weed control strategies aiming to reduce seed bank in the soil via mechanic seed capture at harvest. With the explosion of herbicide resistance evolution, adoption for non-chemical weed control (e.g. mechanical weed seed harvester and destructor) is on the increase, and hence weed biotypes adapting to this practice will eventually evolved.

Conclusion

This study identified and cloned a gene (*PfAG5*) and its interacting proteins that are evidently involved in early-flowering regulation in an ACCase inhibitor resistant *P. fugax* population. This information is useful for better understanding of adaptation to abiotic (herbicide) stress, pleiotropic effect of herbicide resistance evolution in weedy plants, and helpful for better integrated weed management.

Methods

Plant material and growth conditions

Seeds of a *P. fugax* population resistant to ACCase-inhibiting herbicides (referred to as R population) were collected from Qingsheng County (29° 54’ 1” N, 103° 48’ 57” E), Sichuan Province, China, where *clodinafop-propargyl* has been used for over five years and failed to control *P. fugax* in wheat and canola. A susceptible population of *P. fugax* (referred to as S population) were collected from a non-cultivated area in Xichang City of Sichuan (27° 50’ 56” N, 102° 15’ 53” E), where herbicides have never been used. The original R and S populations of *P. fugax* were identified by Dr. Wei Tang (China National Rice Research Institute) and Dr. Fengyan Zhou (Anhui Academy of Agricultural Sciences) [20], and these populations were obtained from the wild without any specifically permissive requirement and now are deposited in the specimen room of Anhui Academy of Agricultural Sciences. The current experiments were conducted in the glasshouse of Anhui Academy of Agricultural Sciences, and no specific permissions were required for samples collection. We stated that the field studies were in accordance with local legislation and no specific licenses were required.

Seeds of the fourth generations of the R and S populations were generated by self-crossing and used in this study. After germination, the seedlings were transplanted into individual 1-L pots containing potting medium (1:1:1:2 vegetable garden soil/compost/ peat/dolomite, pH 6.3). Plants were grown in a glasshouse with average day/night temperatures of 20/10°C under natural sunlight.

Arabidopsis thaliana (L.) Heynh Columbia (*Col*) was obtained from the SALK collection (http://signal.salk.edu/), and used as the wild-type (WT) for transgenic manipulation. The transformed and untransformed control Arabidopsis seedlings were transplanted into individual 0.25L pots containing potting medium (4:1:1 sphagnum/vermiculite/perlite) and grown at 19°C under 100 μmol m^{-2} s^{-1} photo

density of cool white fluorescent light with a photoperiod of either 16/8 h (long day condition, LD), or 8/16 h, light/dark (short day, SD).

Cloning of the PfAG5 cDNA from P. fugax

Total RNA from *P. fugax* R and S plants were isolated using the SGTriEx Total RNA extract Kit (SinoGene), and then used for reverse transcription by Thermo First cDNA Synthesis Kit (SinoGene) according to manufacturer's instructions. The *PfAG5* cDNA fragment was amplified using the primer pair S1 and S2 based on the contig sequence (Table 2), ligated into the pMD18-T vector, and confirmed by sequencing as partial sequence of an *AGAMOUS*-like gene. The full-length coding sequence of the *PfAG5* gene was obtained using 5'-RACE and 3'RACE with the gene-specific primers GSP1 and GSP2 (Table 2) (Clontech, US), and amplified from plants by the primers FK and RB (Table 2) which include introduced *Hind* III and *EcoR* I restriction sites based on the known 5' and 3' sequences.

Table 2
Primers used in the study

Primer	Sequences (5'-3')	Purpose
S1	AATGAGCATGATGACCGATTTGAGC	Clone cDNA fragment
S2	GTTGAAGGGCTGCTGGCCGAGCTG	
GSP1	GGTGTCACTGTTGGCCTTTTTGTACCTC	5'RACE and 3'RACE
GSP2	GAGATCAAGCGCATCGAGAACACCAC	
FK	GGGGTACCATGAGCATGATGAGCATGATGACCG	Clone full-length cDNA fragment
RB	CGGGATCCCTAGTTGAAGGGCTGCTGGCC	
pfAG5-F	CATGGAGGCCGAATTCATGAGCATGATGAGCATGATGACC	Bait vector construction
pfAG5-R	GCAGGTCGACGGATCCCTAGTTGAAGGGCTGCTGGCCGAGC	
ACTIN8-F	CGTCCCTGCCCTTTGTACAC	Reference gene for Arabidopsis
ACTIN8-R	CGAACACTTCACCGGATCATT	
FLC-F	GCTCTTCTCGTCGTCTCC	Analysis of *Flowering locus C* gene expression in Arabidopsis
FLC-R	GTTCGGTCTTCTTGGCTC	
CO-F	AAGGTGATAAGGATGCCAAGGAG	Analysis of *Constans* gene expression in Arabidopsis
CO-R	GGAGCCATATTTGATATTGAACTGA	
SOC1-F	TCAGAACTTGGGCTACTC	Analysis of *Suppressor of over- expression of CO1* gene expression in Arabidopsis
SOC1-R	TTCTCGTCGTCTCCGCCTCC	
AP1-F	TAAGCACATCCGCACTAG	Analysis of *Apetala 1*gene expression in Arabidopsis
AP1-R	TTCTTGATACAGACCACCC	
FT-F	TGGTGGAGAAGACCTCAGGAAC	Analysis of *Flowering locus T* gene expression in Arabidopsis
FT-R	TGCCAAGCTGTCGAAACAATAT	
LFY-F	TGTGAACATCGCTTGTCGTC	Analysis of *LEAFY* gene expression in Arabidopsis
LFY-R	TAATACCGCCAACTAAAGCC	
EF1-F	GAACCTCCCAGGCTGATTGT	Reference gene for *P. fugax*
EF1-R	CAAGAGTGAAAGCAAGAAGAGCA	
pfAG5-F	CAGGCTGGAGAAAGGCATAG	Analysis of *pfAG5* expression in *P. fugax*

Primer	Sequences (5'-3')	Purpose
pfAG5-R	GGAGCTCCATTTCCCTCTTC	
AD1-F	GCTGAAACAGCAGGAGAAGG	Analysis of *AD1* expression in *P. fugax*
AD1-R	AGTCAGCTCCTTAGCCACCA	
AD2-F	CCAGTGAAACAGCAGTGAGC	Analysis of *AD2* expression in *P. fugax*
AD2-R	TGCCTTCTGGTTCTGATGTG	
AD3-F	AGGTCACTGCAGGAGGAGAA	Analysis of *AD3* expression in *P. fugax*
AD3-R	GGCTTGTTGTGTTTGGGTCT	

Molecular characterization and phylogenetic analysis of PfAG5

The open reading frame (ORF) of *PfAG5* cDNA sequence was identified using the ORF finder software (https://www.ncbi.nlm.nih.gov/orffinder/). For homology analysis, the amino acid sequence of *PfAG5* was aligned and compared with the sequences of other species. Phylogenetic analysis was conducted using the neighbor-joining method implemented in MEGA software version 5.0, and the robustness of the inferred phylogeny was validated by including 1000 bootstrap replicates.

Plasmid Construction And Arabidopsis Transformation

The pCAMBIA2300 and pCAMBIA1303 plasmid vectors were digested by *Hind* III and *EcoR* I, respectively. The (*CaMV*) *35S* promoter of pCAMBIA2300 (1008 bp) and the large skeleton of pCAMBIA1303 were recovered and purified. Then, T4 DNA ligase (TaKaRa) was used to connect the two parts and a new two-element expression vector pCAMBIA1303-*35S:35ST* including the 35S promoter was obtained.

The full-length ORF of *PfAG5* gene was ligated into the binary vector pCAMBIA1303-*35S:35ST* (empty plasmid control, *Mock*) to generate the plasmid pCAMBIA1303-*35S-35ST:PfAG5* (Fig. 6A). The plasmid was transferred into WT Arabidopsis plants (*Col*) using the floral dipping method. All transgenic Arabidopsis seeds (T_0) were screened on 1/2 MS solid medium containing 50 mg-L^{-1} hygromycin. Positive transgenic lines (T_1, n = 40) were confirmed by PCR amplification of the hygromycin gene, and the target gene (*PfAG5*) was visualized by the GUS gene histochemical localization (Fig. 6B). Introduction of the target gene (*PfAG5*) in T2 generation plants was verified by PCR, and positive plants (n = 27) all showed an early flowering phenotype. Twenty of these lines were used to produce the T3 generation and were used in the following experiments.

Flowering Time And Seed Production Measurements

To measure flowering time, seeds of WT (*Col*), empty plasmid control (*Mock*) and *PfAG5* transgenic Arabidopsis plants (35S::*PfAG5*) were surface sterilized with 10% hypochlorite, then placed on MS agar medium and stratified at 4°C for 48 h before being placed at 19°C. Ten-day-old seedlings (at the four leaf

stage) were transferred to growth medium (1:4:1 of vermiculite, sphagnum and perlite), and grown under LD or SD conditions.

The flowering time of 20 T3 transgenic lines were recorded from the day of transplanting until the first Arabidopsis flower bloomed. Rosette leaf numbers were recorded when peduncle was 1–2 cm in length, and above-ground plant height and pod numbers were determined on day 55 after transplanting. Seeds were collected on day 62 after transplanting, and weighed after drying at 37°C for 24 h.

Yeast Two-hybrid Assay

Above-ground plant tissue of three R *P. fugax* plants at early flowering stage were harvested randomly, and the cDNA library (cloned into Prey vector pGADT7) was obtained using the Clontech kit (catalog number 630490). The full-length *PfAG5* (with yeast codon optimization) was cloned into vector pGBKT7 (Bait vector) and then transformed into the yeast strain Y2HGold (Clontech).

The Matchmaker™ Gold Yeast Two-Hybrid System (Clontech, US) was used to screen the *PfAG5* interaction proteins from the R *P. fugax* library according to the manufacturer's instructions. The primers used for pGBKT7 vector construction were listed in Table 2. To confirm the interactions, the identified Prey and Bait vectors were validated by one-to-one interaction hybridization.

PfAG5 expression analysis in Arabidopsis and P. fugax

To analyze the expression pattern of *PfAG5* in different tissues of transgenic Arabidopsis plants, leaf, flower and pod samples from 3–5 T3 lines were collected at the seedling (6–8 leaves), flowering (full open) and podding (new formation) stages. Harvested samples were snap frozen in liquid nitrogen and stored at -80°C until use. In addition, the whole above ground part of *PfAG5* transgenic and WT Arabidopsis plants were collected before midday (Zeitgeber time 6, ZT6) at the flowering stage (13and 28 d after transplanting, respectively) for analysis of the expression patterns of six other Arabidopsis genes relevant to flowering regulation: *CO*, *SOC1*, *FT*, *LFY*, *FLC* and *AP1*.

Tissue samples of the R and S *P. fugax* plants were collected at the seedling and tillering stages, and the samples collected at the early flowering stage of R plants correspond to the heading stage of the S plants. The expression of *PfAG5* and interacting proteins (AD-1, AD-2 and AD-3) were compared between R and S samples which were collected at the same time.

Total RNA was extracted using the SGTriEx Total RNA extract Kit (SinoGene), and DNA contamination removed by RNase-free DNase (Fermentas). The DNA-free RNA was then used for reverse transcription by Thermo First cDNA Synthesis Kit (SinoGene). The primer sequences used for Real-time quantitative PCR are provided in Table 2. The *ACTIN8* and *EF1* gene was used respectively for normalization of Arabidopsis and *P. fugax* samples. The qPCR amplification was conducted for up to 40 cycles using the following thermal profile: denaturation at 95°C for 15 s, annealing at 55°C for 15 s and extension at 72°C

for 45 s. The RT-qPCR results were presented as means ± SE of three biological replicates each performed in triplicate. Gene expression level was estimated as $2^{-\triangle\triangle Ct}$.

Abbreviations

FLC, Flowering locus C; *CO*, Constans; *SOC1*, Suppressor of over-expression of *CO1*; *AP1*, Apetala 1; *FT*, Flowering locus T; *LFY*, LEAFY; *FRI*, FRIGIDA.

Declarations

Ethics approval and consent to participate

Not applicable

Consent to publish

Not applicable

Availability of data and materials

The identified *PfAG5* sequence in this paper has been deposited in the GenBank (accession no. MK559453). Experimental materials are available upon request by qualified researchers to the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

FYZ, QY and YZ conceived and designed the experiments. FYZ, CCY, YJH and YS performed the experiments. FYZ and QY wrote the article. All authors read and approved the final manuscript.

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Figures

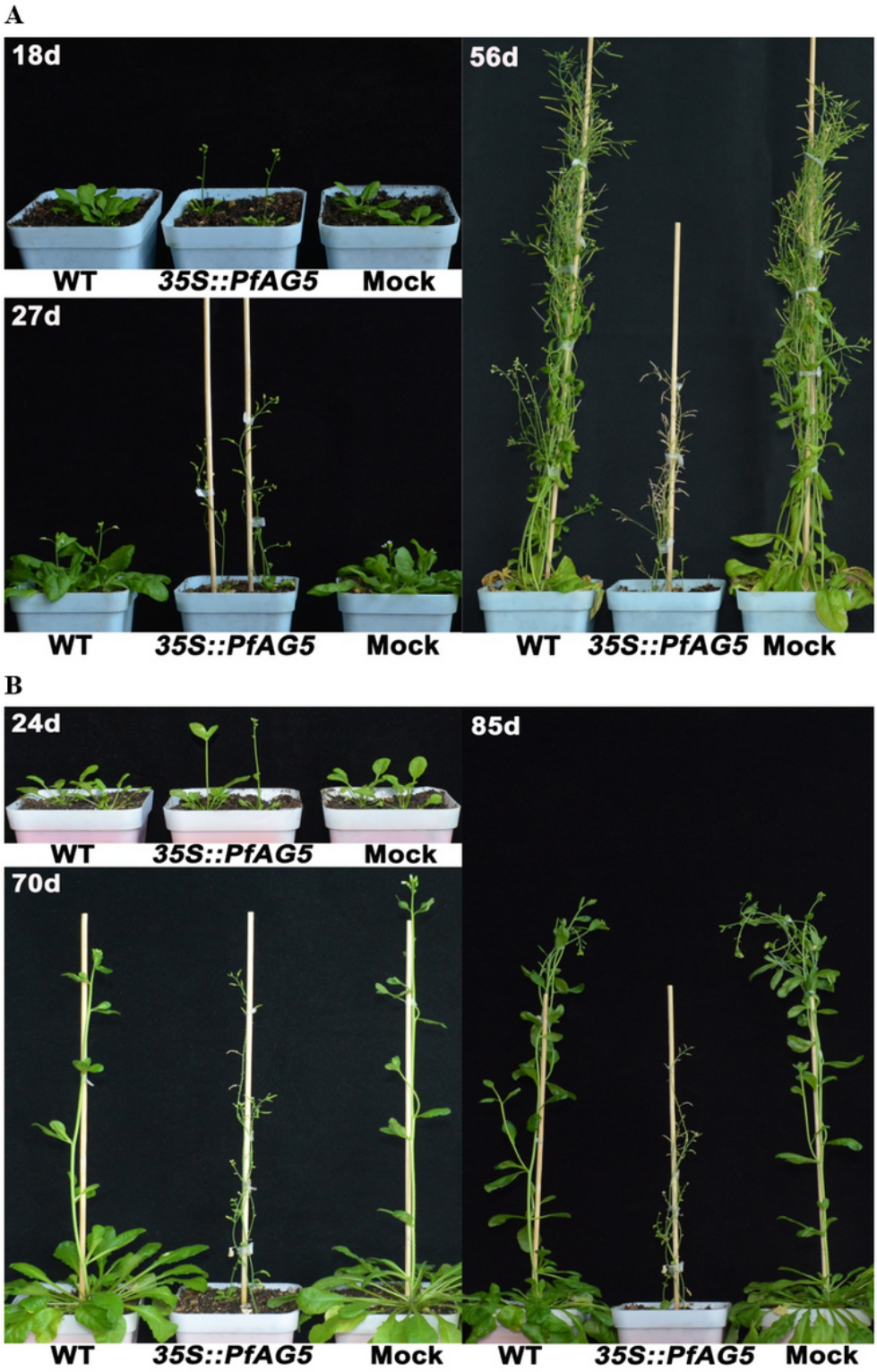


Figure 1

Representative flowering phenotypes of PfAG5 transgenic Arabidopsis plants under long day (LD) (A) and short day (SD) conditions (B). Photos were taken 18, 27 and 56d after transplanting under LD conditions, and 24, 70 and 85d after transplants under SD conditions.

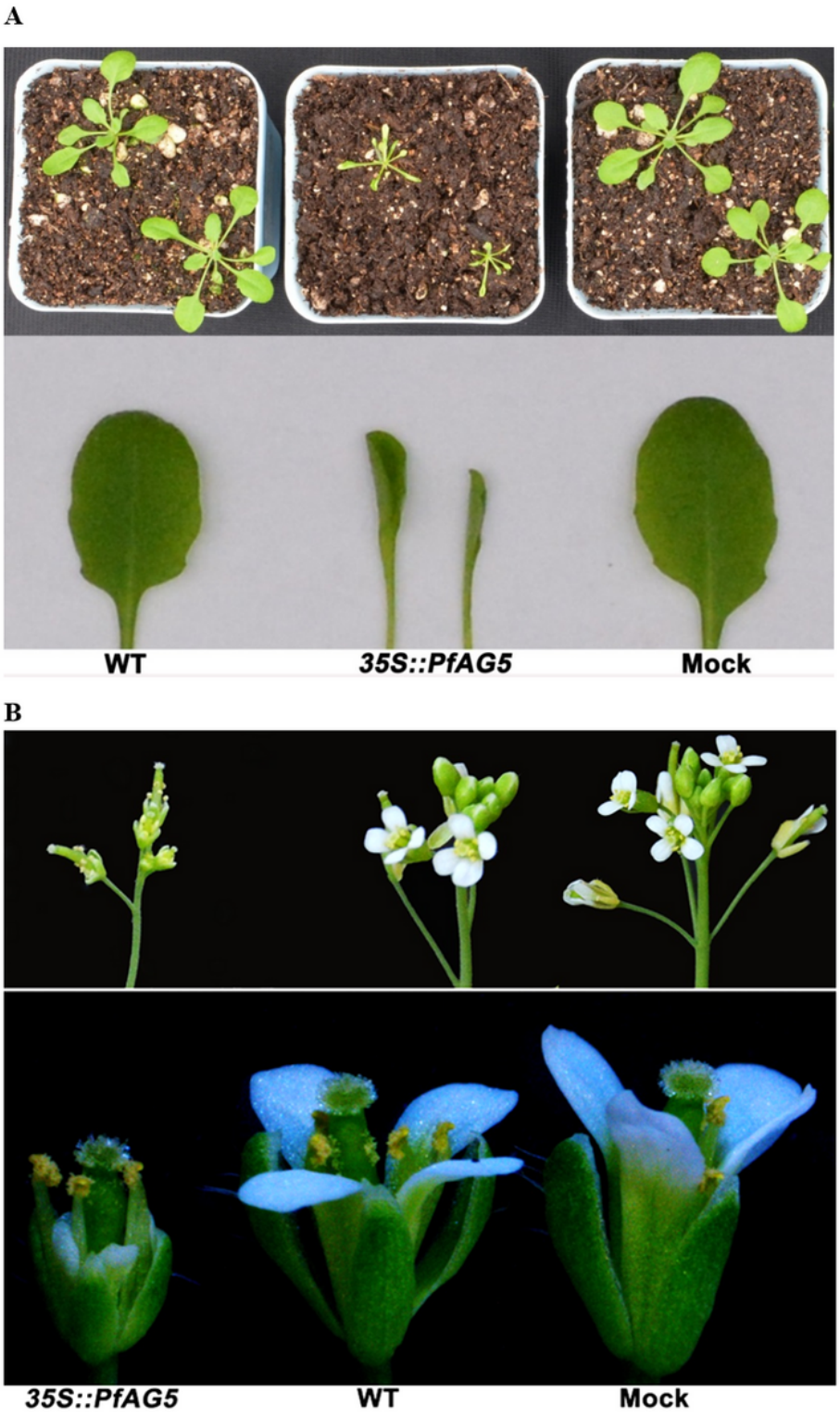


Figure 2

Representative images showing phenotypic changes of PfAG5 transgenic Arabidopsis plants compared with the controls (WT and Mock). A. Phenotypic differences in leaves of PfAG5 transgenic plants 14 d after transplanting. B. Flower phenotype diversity of PfAG5 transgenic plants.

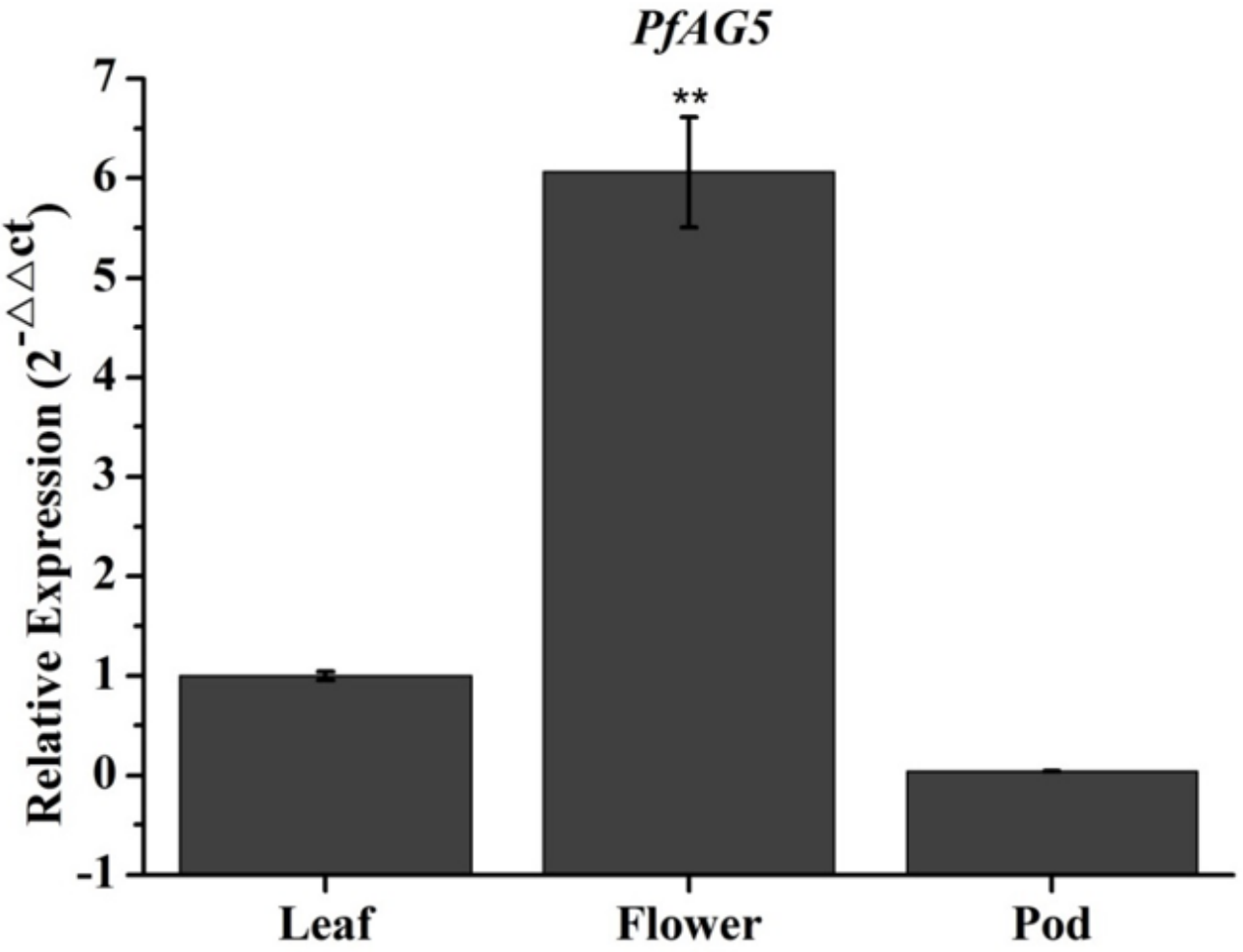


Figure 3

RT-qPCR analysis of PfAG5 gene expression in different tissues of Arabidopsis transgenic plants (35S::PfAG5). **indicates significant difference, P < 0.01.

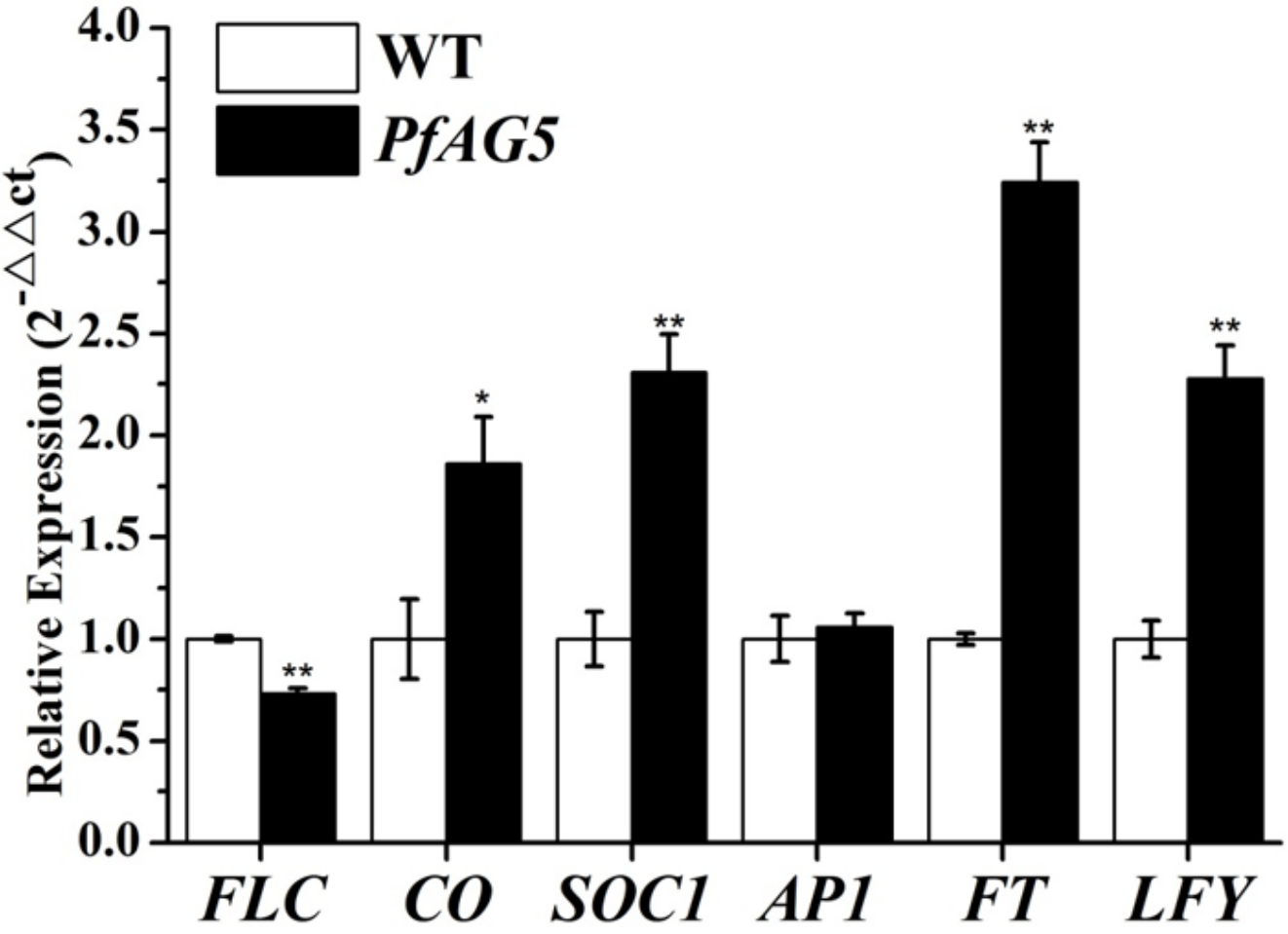


Figure 4

RT-qPCR analysis other endogenous flowering-related genes in WT and PfAG5 Arabidopsis transgenic plants (above-ground materials) at the flowering stage. The ACTIN8 gene was used as an internal control. The transcript level in WT was set as the standard, with a value of 1. * indicates significant difference p<0.05□** Significant difference P<0.01. FLC, Flowering locus C; CO, Constans; SOC1, Suppressor of over-expression of CO1; FT, Flowering locus T; LFY, LEAFY.

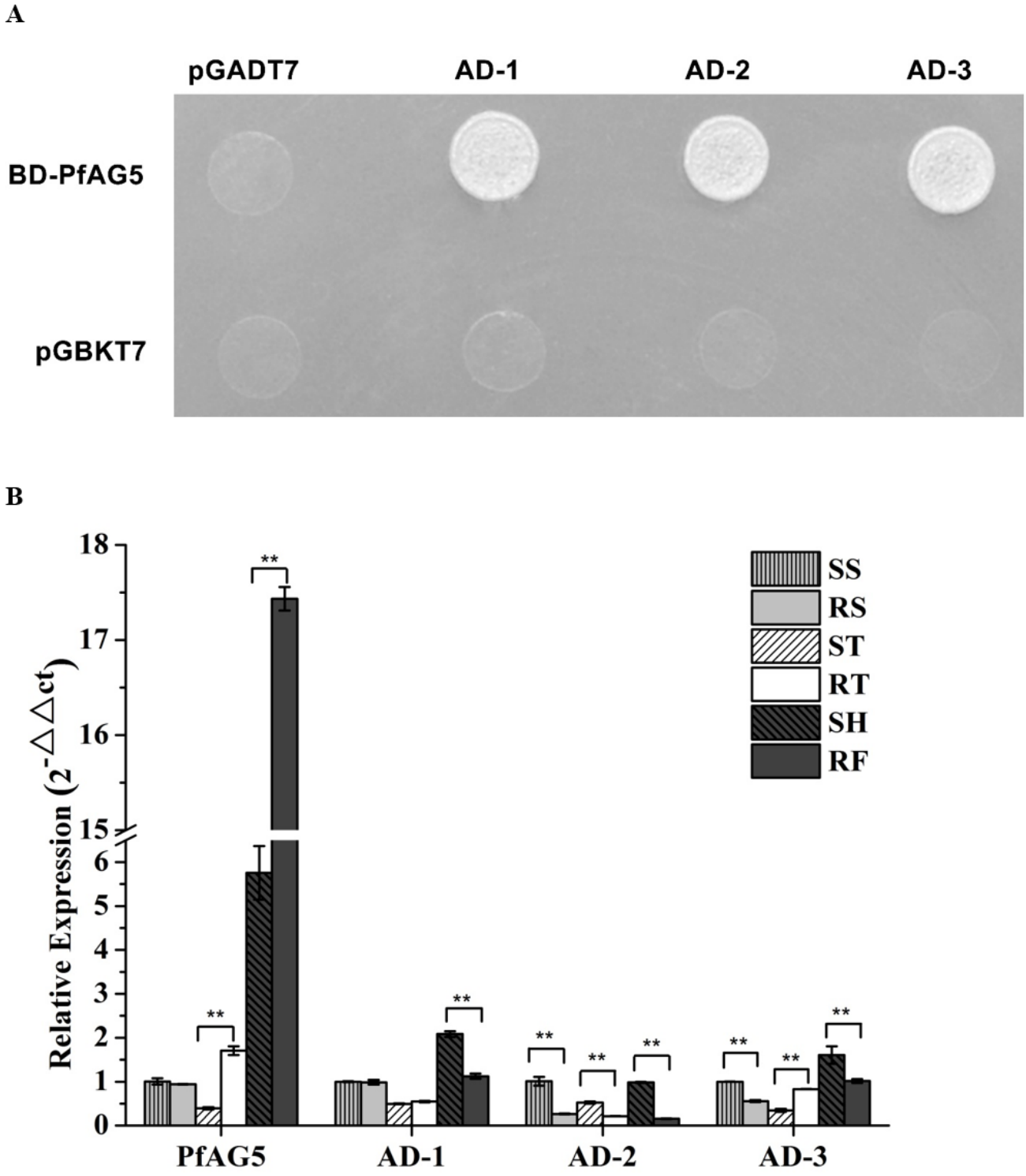


Figure 5

Assays of the yeast two-hybrid and expression. (A) Protein interaction was indicated by the ability of yeast cells to grow on synthetic dropout medium lacking Leu/Trp/His/Ade. Genes for PfAG5 and three interaction proteins identified were cloned into pGBKT7 (shown as BD) and pGADT7 (shown as AD), respectively. (B) RT-qPCR analysis of PfAG5 and three interaction protein genes in the resistant population of P. fugax. ** indicates significant difference $p<0.01$. SS: Susceptible plants at the seedling

stage; RS: Resistant plants at the seedling stage; ST: Susceptible plants at the tillering stage; RT: Resistant plants at the tillering stage; SH: Susceptible plants at the heading stage; RF: Resistant plants at the flowering stage.

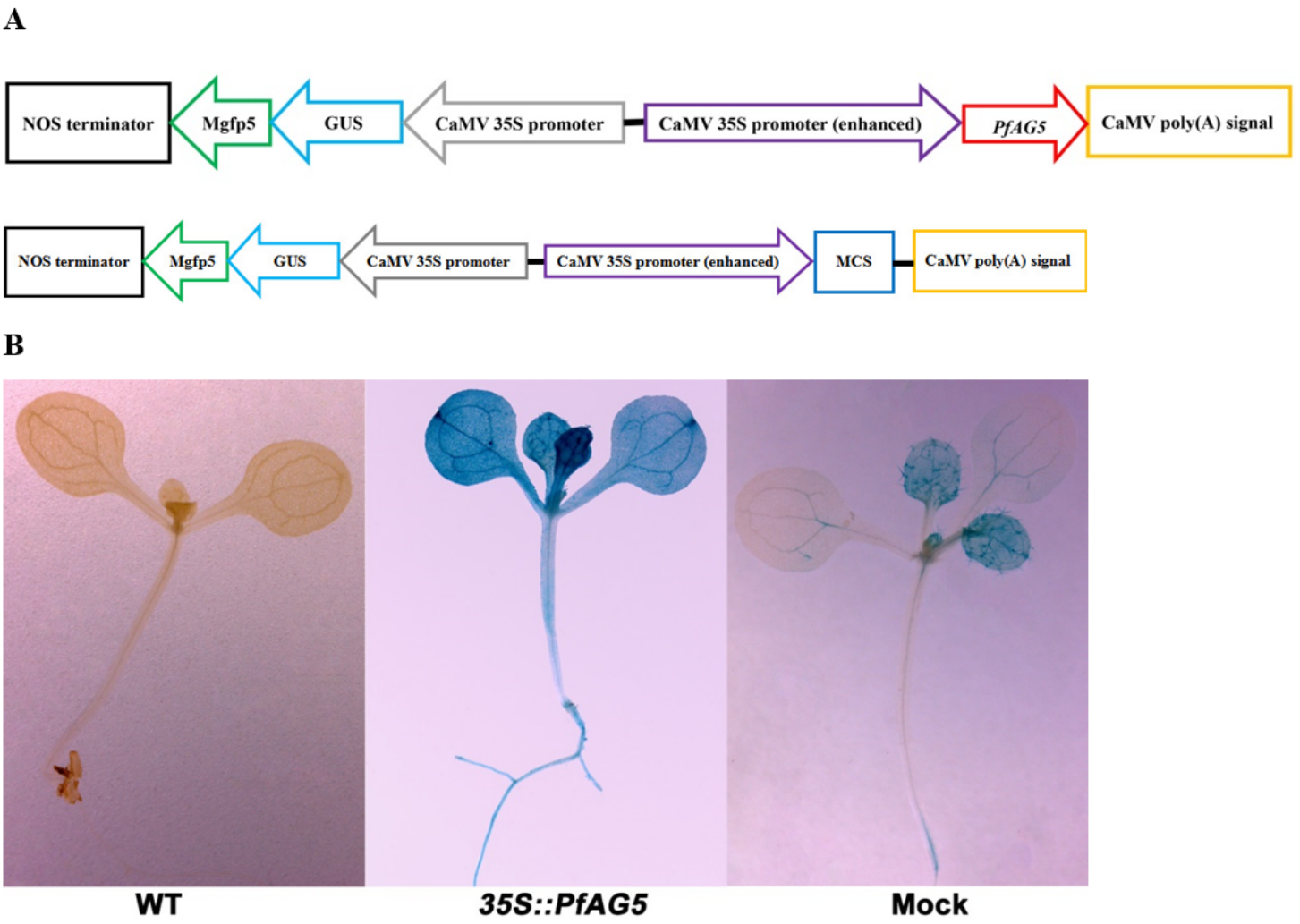


Figure 6

Construction of PfAG5 transgenic vector and positive lines verification (A) Schematic diagram of the T-DNA region of the binary plasmid (PfAG5 and Mock) used in this study. (B) Expression patterns of the pfAG5 gene in representative transgenic Arabidopsis plants. Plants were grown under long day conditions for 8 d and the whole plants used for GUS staining. WT, wild-type; 35S::pfAG5, PfAG5 transgenic Arabidopsis; Mock, empty plasmid control.

Supplementary Files

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