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

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Abstract. Herbicides are the major tool for controlling large populations of yield depleting weeds. However, over-reliance 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. In this study, a homologue gene of *AGAMOUS* sub-family (referred to as *PfAG5*) of the MADS-box family was cloned from plants of an early flowering *Polypogon fugax* Nees ex Steud. population resistant to the ACCase inhibitor herbicide (clodinafop-propargyl). The *PfAG5* gene was functionally characterised in *Arabidopsis thaliana* L. Overexpression of the *PfAG5* gene in *Arabidopsis* resulted in early flowering, abnormal flowers (e.g. small petals), short plants and reduced seed set, compared with 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 expressed earlier and higher in the resistant (R) than the susceptible (S) *P. fugax* plants. Furthermore, one protein (FRIGIDA-like) with relevance to flowering time regulation and interacts with *PfAG5* in resistant (R) *P. fugax* was identified by the yeast two-hybrid and pull-down assays. These results suggest that the *PfAG5* gene is involved in modulating early flowering in *P. fugax*.

Keywords: *Polypogon fugax* Nees ex Steud., *Arabidopsis thaliana* L., herbicide resistance, early flowering, flowering regulation, *AGAMOUS*-subfamily.

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Introduction

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 flowering time is critical for flowering plants (Wei *et al*. 2016). Flowering time is regulated autonomously and by environmental factors, such as photoperiod, vernalisation and stresses (Takeno 2016).

Many stress factors have been reported to induce flowering, such as light intensity, UV light, temperature, nutrition and drought, as summarised in previous reviews (Wada and Takeno 2010; Kazan and Lyons 2016). Herbicide application is also a stress to weedy plants, and it can select for evolution of herbicide resistance (Powles and Yu 2010). Adaptive changes in seed germination and seedling emergence, flower bud formation and flowering time have also been observed in herbicide resistant biotypes (Wang *et al*. 2010; Kleemann and Gurjeet 2013; Owen *et al*. 2014; Babineau *et al*. 2017; Kaspary *et al*. 2017).

In agriculture, frequent and regular disturbances from ploughing and harvesting likely exert a strong selection on weeds for rapid flowering and seed set (Barrett 1983). 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* L. exhibited significant standing genetic variations to adapt to flowering time selection (Ashworth *et al*. 2016). 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 (Hill *et al*. 2016).

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 (Masiero *et al*. 2011). In *Arabidopsis thaliana* L., four genes, *AGAMOUS* (*AG*), *SHATTER*

PROOF1 (*SHP1*), *SHP2* and *SEEDSTICK* (*STK*) compose the monophyletic *AG*-subfamily within the MADS-box gene phylogeny (Favaro *et al*. 2003; Kramer *et al*. 2004). Members of the *AG*-subfamily are involved in the specification of floral reproductive organs and required for normal development of carpels and fruits (Dreni and Kater 2014). For instance, when expressed in *Arabidopsis* the *AG*-subfamily genes from *Gossypium hirsutum* L. regulate flower development and fruit formation (de Moura *et al*. 2017). 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 functions by influencing 'meristem identity genes', which control the transition from inflorescence to floral meristems (Becker and Theißen 2003).

In our previous study, we found that the resistant (R) population of *Polypogon fugax* Nees ex Steud.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 (S) population (Tang *et al*. 2014). Plants of the R *P. fugax* population were found to be earlier in head emergence, flowering and seed maturation than that of the S population (Tang *et al*. 2015). 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* (Zhou *et al*. 2017). 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 in *Arabidopsis*. We analysed the expression pattern of *PfAG5* gene in transgenic *Arabidopsis* and R versus S *P. fugax* plants, and that of other six endogenous flowering regulation genes in *Arabidopsis*. Furthermore, we identified two *PfAG5* interaction proteins in R *P. fugax* by the yeast two-hybrid and pull-down assays, and one (FRIGIDA-like) is reverent to flowering time regulation. This is among the very few studies on the regulation mechanism of early flowering in a weedy plant species (Zhou *et al*. 2020). This knowledge will aid in future genetic approaches for better weed control strategies.

Materials and methods

Plant material and growth conditions

Seeds of a *Polypogon fugax* Nees ex Steud. population resistant to ACCase-inhibiting herbicides (referred to as R population) were collected from Qingsheng County (29°54′N, 103°48′E), Sichuan Province, China, where *clodinafop-propargyl* has been used for over 5 years and failed to control *P. fugax* in crops of wheat (*Triticum aestivum* L.) and canola (*Brassica napus* L.). 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′N, 102°15′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) (Tang *et al*. 2014), and these populations were obtained from wild populations without any specifically permissive requirement and now are deposited in the specimen room of Anhui Academy of Agricultural Sciences.

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. cv. Columbia (Col-0) 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 0.25 L 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 light/dark (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 1), ligated into the pMD18-T vector, and confirmed by sequencing to be the partial sequence of an *AGAMOUS*-like gene. The full-length coding sequence of the *PfAG5* gene was obtained using 5′- and 3′-RACE with the gene-specific primers GSP1 and GSP2 (Table 1) (Clontech, US), and amplified from plants by the primers FK and RB (Table 1) with introduced *Hind*III and *EcoR* I restriction sites based on the known 5′ and 3′ sequences.

Molecular characterisation 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 neighbour-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. T4 DNA ligase (TaKaRa) was then used to connect the two parts and a new two-element expression vector

Table 1. 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 overexpression 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*
pfAG5-R	GGAGCTCCATTTCCCTCTTC	
IRP1-F	GCTGAAACAGCAGGAGAAGG	Analysis of *IRP1* expression in *P. fugax*
IRP1-R	AGTCAGCTCCTTAGCCACCA	
IRP3-F	AGGTCACTGCAGGAGGAGAA	Analysis of *IRP3* expression in *P. fugax*
IRP3-R	GGCTTGTTGTGTTTGGGTCT	

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* (see Fig. S3*a*). 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 visualised by the GUS gene histochemical localisation (see Fig. S3*b*). 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 lines 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 sterilised 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 vermiculite:sphagnum: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 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

Aboveground plant tissue of three R *P. fugax* plants at the early flowering stage were harvested randomly, and the cDNA library (cloned into Prey vector pGADT7) was obtained using the Clontech kit (catalogue number 630490). The full-length *PfAG5* (with yeast codon optimisation) 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 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 1. To confirm interactions, the identified Prey and Bait vectors were validated by one-to-one interaction hybridisation.

Pull-down assay

For the *in vitro* interaction assays, The CDS of *PfAG5* was reconstructed into the GST pull-down pET28a vector, which then used to transform Top10 *Escherichia coli*. Single colonies of GST-PfAG5 were inoculated in LB medium and inoculated at 37°C until OD600 reached 0.6–0.8. After induction with 0.5 mM IPTG at 37°Cfor 4 h, cells were collected and resuspended in PBS buffer. The aboveground part of R *P. fugax* plants at the early flowering stage was ground in liquid nitrogen to extract proteins for the pull-down assay according to Dou *et al.* (2019). The treated samples were then analysed by liquid mass spectrometry (LC–MS/MS) (Ultimate 3000 and Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer; Thermo Fisher Scientific) and proteins were identified by peptide sequencing. The peptide sequences combined with the peptide mass were then used to search against a protein sequence database for identification of candidate interaction proteins. MaxQuant (1.6.2.10) was used to search the Uniprot protein library to obtain potential interaction proteins, which were annotated using the Gramineous Genome Database (see Table S1).

PfAG5 *expression analysis in* Arabidopsis *and* P. fugax

To analyse the expression pattern of *PfAG5* in different tissues of transgenic *Arabidopsis* plants, leaf, flower and pod samples from three to five 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 aboveground part of *PfAG5* transgenic and WT *Arabidopsis* plants were collected before midday (Zeitgeber time 6, ZT6) at the flowering stage (13 and 28 days 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 its interacting proteins 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 DNaseI(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 (real-time qPCR) are provided in Table 1. The *ACTIN8* and *EF1* gene was used respectively for normalisation 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 real-time qPCR results were presented as means ± s.e. of three biological replicates each performed in triplicate. Gene expression level was estimated as 2^{-Ct}.

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* L. ssp. vulgare AGAMOUS-like protein 1 HvAG1 (AAL93196.1) and *Aegilops tauschii* Coss. MADS box transcription factor WAG-2f (ALM58837.1). A phylogenetic tree was constructed to determine the relationship of PfAG5 protein with AGAMOUS-like proteins of other plant species. PfAG5 belongs to the family of AGAMOUS homologues from monocots and is closely related to AGAMOUS-like proteins from *H. vulgare* ssp. vulgare, *T. aestivum* and *A. tauschii* (see Fig. S1*a*). Sequence alignment revealed that *PfAG5* has a conserved DNA-binding SRF-type TF domain, MADS-box domain and AG Motif (see Fig. S1*b*).

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 2; Fig. 1*a*). Under short day (SD) conditions, *PfAG5* transgenic plants flowered ~50–60 days

Table 2. Changes in growth and reproduction of *Arabidopsis thaliana* L. overexpressing the *PfAG5* gene under long day or short day conditions
Data are mean ± s.e. (*n*, 20 lines). Different letters in a column indicate significant difference by Duncan's multiple comparison test, $P < 0.01$

Treatment	Rosette leaves	Flower time (day)	Pod numbers	Plant height (cm)	Seed yield (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}

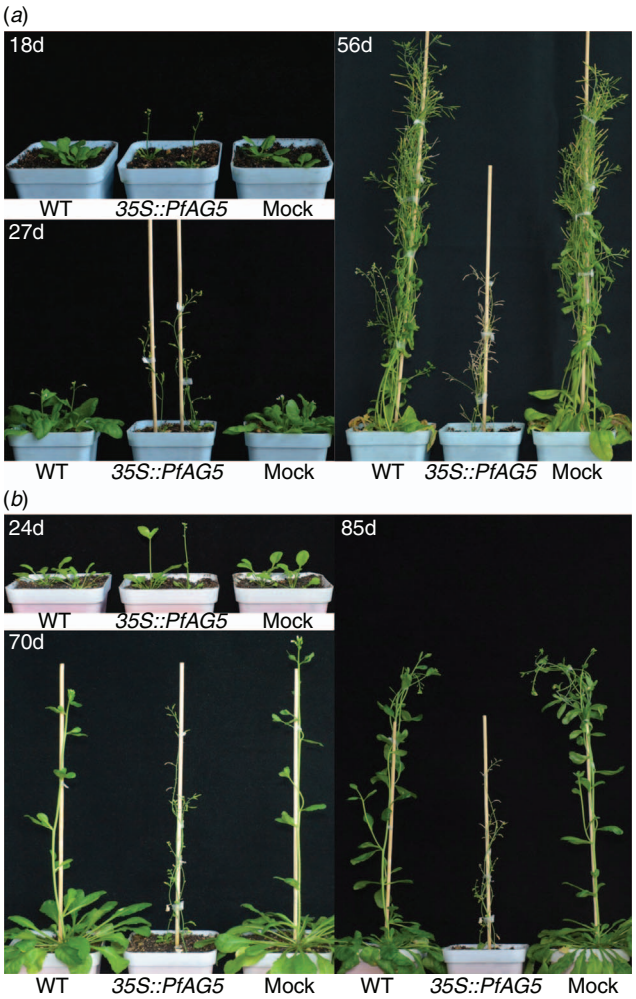

Fig. 1. Representative flowering phenotypes of *35S*::*PfAG5* under (*a*) long day (LD) and (*b*) short day (SD) conditions. Photos were taken 18, 27 and 56 days after transplanting under LD conditions, and 24, 70 and 85d after transplants under SD conditions.

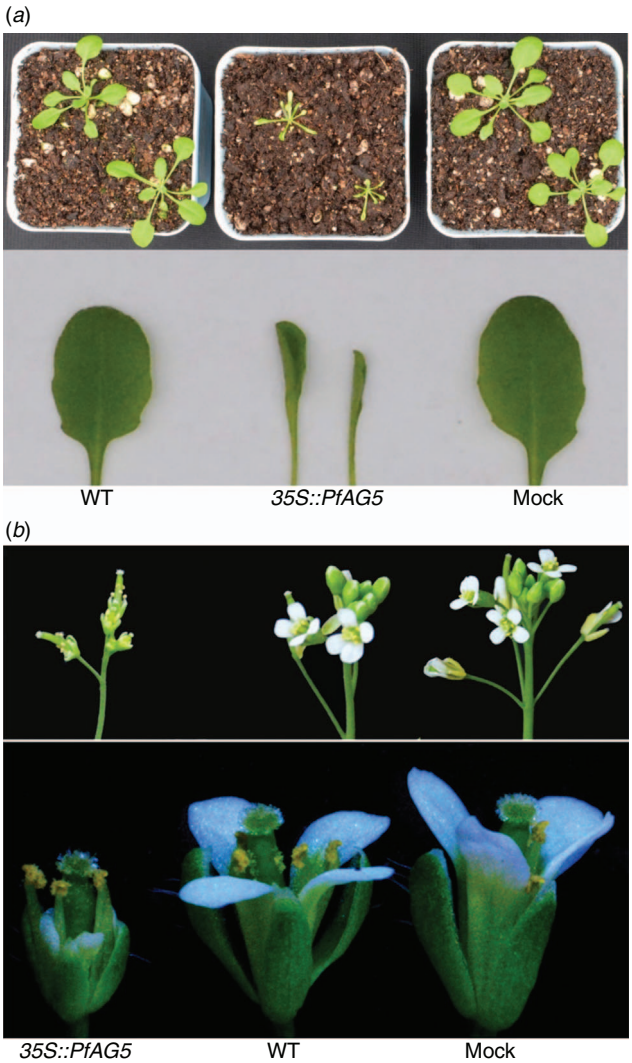

Fig. 2. Representative images showing phenotypic changes of *35S*::*PfAG5* compared with the controls (WT and Mock). (*a*) Phenotypic differences in leaves of *35S*::*PfAG5* 14 days after transplanting. (*b*) Flower phenotype diversity of *PfAG5* transgenic *Arabidopsis* plants.

earlier and produced 35–38 fewer rosette leaves than control plants (Table 2; Fig. 1*b*).

In contrast to control plants, *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 (see Fig. S2). However, plant height, pod number and seed yield were all lower in *PfAG5* transgenic plants than in WT and Mock controls (Table 2; Fig. 1). Thus, expression of *PfAG5* in *Arabidopsis* resulted in the early flowering phenotype with abnormal flowers and reduced seed set.

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 analysed by real-time qPCR. Results showed that *PfAG5* was constitutively expressed in leaves and flowers, and the expression level was significantly higher in leaves and flowers but lower in young pods than in controls (Fig. 3). This is similar to the MADS-box gene *BdMADS33* of *Brachypodium distachyon* L., which showed weak expression signals in young seeds (Wei *et al*. 2014).

In *PfAG5* transgenic *Arabidopsis* plants, higher expression of *Arabidopsis* endogenous genes such as *CONSTANS* (*CO*), *SUPPRESSOR OF CONSTANS OVEREXPRESSION1* (*SOC1*), *Flowering locus T* (*FT*), *LEAFY* (*LFY*), and lower expression of the *FLOWERING LOCUS C* (*FLC*) gene, were found in comparison to WT, in the whole aboveground 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 interacting with *PfAG5* in R *P. fugax* (named as IRP1, IRP2 and IRP3) were identified by the yeast two-hybrid

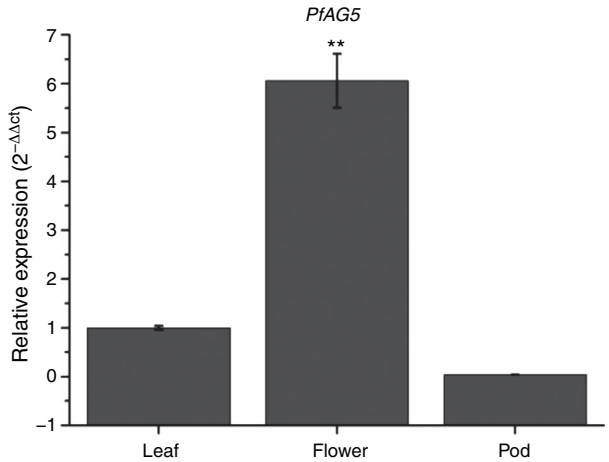

Fig. 3. Real-time qPCR analysis of *PfAG5* gene expression in different tissues of transgenic *Arabidopsis* plants (*35S*::*PfAG5*). **, $P < 0.01$.

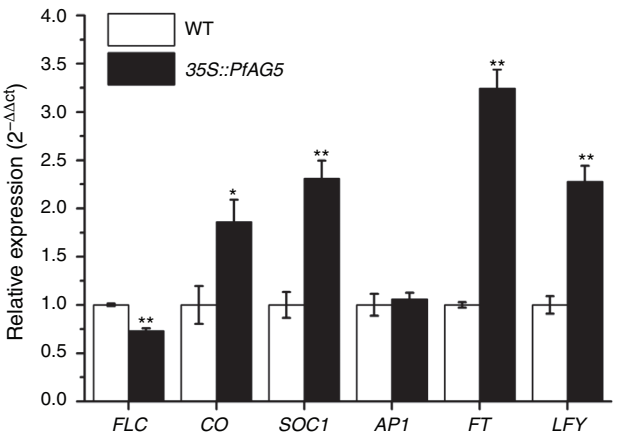

Fig. 4. Real-time qPCR analysis of other endogenous flowering-related genes in WT and *35S*::*PfAG5* transgenic *Arabidopsis* plants (aboveground materials) at the flowering stage. The *ACTIN8* gene was used as an internal control. The transcript level in WT was set as the standard, with a value of 1. *, $P < 0.05$; **, $P < 0.01$.

system (Fig. 5*a*). IRP1 showed 91% amino acid sequence identity to *A. tauschii* ssp. *tauschii* FRIGIDA-like protein 3 (XM_020321692), IRP2, 82% identity to *A. tauschii* ssp. *tauschii* *AGAMOUS*-like MADS-box protein AGL66 (XM_020339220.1), and IRP3, 93% identity to MADS2 (AY198327.1) of *Lolium perenne* L. and fruitful-like MADS-box transcription factor (FUL2) (DQ792967.1) of *Avena sativa* L.

In the pull-down assay, two interaction proteins associated with flowering time regulation were identified: IRP1 (FRIGIDA-like protein (A0A453KI48, uniport protein ID)) with 100% identity to *A. tauschii* ssp. tauschii FRIGIDA-like protein 3 (XM_020321692) and IRP3 (wheat MADS-box transcription factor TaAGL29 (A0A3B6AZ67, uniport protein ID)) with 100% identify to *L. perenne* MADS2 (AY198327.1) (see Sequence File S1). Therefore, these two interacting proteins were confirmed by the two independent assays, and hence no further analysis for IRP2 was conducted.

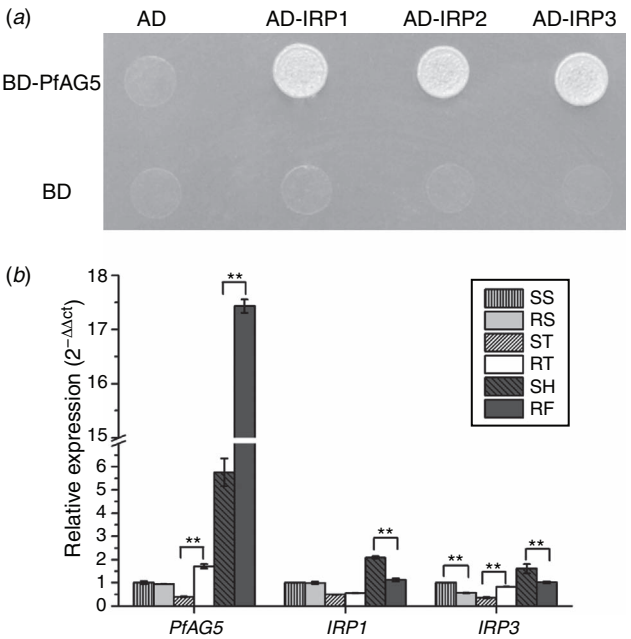

Fig. 5. Identification of PfAG5 interaction proteins and their expression. (*a*) Yeast two-hybrid assay. Protein interaction was indicated by the ability of cells to grow on synthetic dropout medium lacking Leu/Trp/His/Ade. Full-length *PfAG5* and truncated *IRP1*, *IRP2 or IRP3* were cloned into pGBKT7 (named as binding domain vector, BD) and pGADT7 (named as activation domain vector, AD), respectively. (*b*) Real-time qPCR analysis of *PfAG5* and the two interaction protein (which were verified in both yeast two-hybrid and pull-down assays) genes in the resistant population of *P. fugax*. 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. **, $P < 0.01$.

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

The expression patterns of *PfAG5* and the two 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 with the seedling stage. However, *PfAG5* expression was significantly higher in R than the S plants at the tillering and flowering stages (Fig. 5*b*). 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. 5*b*). Conversely, the expression of *IRP1* was 1.86-fold lower at the early flowering stage of R than S plants, while there was no significant differences at the seedling and tillering stages. However, there was no clear pattern in the expression of *IRP3* (Fig. 5*b*).

Discussion

Flowering time of many weedy species is synchronised with that of crops (Tremblay and Colasanti 2007) 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 flowering time (Wang *et al*. 2010; Ashworth *et al*. 2016). For instance, in a glyphosate-resistant population of *Conyza bonariensis* (L.) Cronq. from Brazil, the first floral bud formation was observed 28 days earlier than the glyphosate-susceptible population (Kaspary *et al*. 2017). An ALS herbicide resistant population of *Apera spica-venti* (L.) Beauv. flowered 13 days earlier than the susceptible population at a certain crop density (Babineau *et al*. 2017). Panicles of the ACCase herbicide resistant (due to the 2041 mutation) *Hordeum glaucum* L. biotype emerged ~20 days earlier than that of susceptible biotype in the field (Shergill *et al*. 2016). The ACCase herbicide-resistant (due to the 1781 mutation) plants of *Setaria viridis* (L.) Beauv. flowered and matured earlier but producing 24% more seeds than the susceptible plants (Wang *et al*. 2010).

In our previous study, we found that an ACCase herbicide-resistant (due to the 2041 mutation) population of *P. fugax* reached the tiller and panicle emergence and seed shedding stages 6, 10 and 12 days, respectively, earlier than the S population (Tang *et al*. 2019). Working with this early flowering population, we identify an *AGAMOUS*-subfamily gene *PfAG5* that is likely involved in early flowering in R population in this study. The *AGAMOUS*-like gene subfamily includes members involved in the specification of stamen, carpel and ovule. Phylogenetic analysis showed that *PfAG5* groups into *AGAMOUS*-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* (Wang *et al*. 2015), and *T. aestivum TaAGL39* (Zhao *et al*. 2006). Overexpression of *AGL79* in *Arabidopsis* was found to result in narrow leaf shape, fewer numbers of leaves and early flowering (Gao *et al*. 2018), which is consistent with observed phenotypes in *PfAG5* transgenic *Arabidopsis* plants in the current study (Figs 1, 2*a*).

It is known in *Arabidopsis* that the floral integrator *FT* is a key regulator of flowering time (Komiya *et al*. 2008), and transcription factor *CO* activates the expression of *FT* (Tamaki *et al*. 2007), promoting early flowering. Indeed in our experiment found that the expression of *FT* (3.2-fold) and *CO* (1.9-fold) in transgenic *Arabidopsis* (*35S*::*PfAG5*) were significantly higher than in WT (Fig. 4). So, we speculate that the *PfAG5* gene may promote the expression of *CO* in transgenic plants, and the high expression of *CO* in turn may activate the expression of *FT*. In contrast, the expression of *FLC* can represses the transcriptional activation of the floral integrator genes *FT* and *SOC1* (Helliwell *et al*. 2006), hence inhibiting flowering. In this study, *FLC* expression was inhibited in *PfAG5* transgenic plants, which may release repression of *FT* and *SOC1* and promote flowering (Fig. 4). This can be tested by expressing *PfAG5* in *Arabidopsis FLC*, *SOC1* or *FT* knockout mutants.

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 (Winter *et al*. 2015). It is known that *AP1* plays a role in differentiation of sepals and petals (Pabón-Mora *et al*. 2012). However, in the current study, no difference in the expression of *AP1* was detected in *PfAG5* transgenic *Arabidopsis* relative to WT plants 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* L. (carnation) did not significantly differ in petals of different flower phenotypes (Wang *et al*. 2020). It was found that overexpression of *LFY* resulted in early flowering (Nilsson *et al*. 1998), likely via causing precocious development of flowers, converting the inflorescence shoot into a single terminal flower (Weigel and Nilsson 1995). So the high expression of *LFY* (2.3-fold) in *PfAG5* transgenic *Arabidopsis* plants may be related to early flowering and abnormal flowers (Fig. 4). This conjecture can be further verified in a *LFY* knockout mutant line of *Arabidopsis*.

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 (Kralemann *et al*. 2018). 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 2). *Arabidopsis* plants transformed with carnation *AGAMOUS* genes (*DcaAGa*, *DcaAGb*) also showed petal loss, short silique, and seed sterility (Wang *et al*. 2020), and this is similar to the flower phenotype of *PfAG5* transgenic plants, except for seed viability. These results imply that *PfAG5* gene is likely a flowering time promoter for the efficient expression of other flowering time regulatory genes, causing early flowering and abnormal flowers in *P. fugax*. However, what about the possible flowering regulation pathways of *PfAG5* in R *P. fugax* population?

In this current study, we identified two *PfAG5* interacting proteins (named as IRP1 and IRP3) with homology to *FRI3* and *FUL2* gene, respectively. In *Arabidopsis*, *FRI* causes later flowering by enhancing expression of the flowering repressor gene *FLC* (Michaels and Amasino 2001) and RNA silencing of *FRI*-like protein 3 mRNA (*FRL3*) induces early flowering in plants of *Solanum lycopersicum* L. (tomato) (Adkar-Purushothama *et al*. 2018). Despite the central role of *FLC*, most of the variations in flowering time have been correlated with natural allelic diversity of *FRI* (Michaels and Amasino 1999). For instance, among *FRIGIDA* orthologues, the *BnaA3. FRI* was tightly associated with flowering time variation in *B. napus* (Yi *et al*. 2018). In our study, the *FRI-like* gene (*IRP1*) was inhibited at the flowering stage of R *P. fugax* plants, contrary to the high expression of *PfAG5* (Fig. 5*b*). Therefore, inhibition of the *FRI* gene (*IRP1*) caused by overexpression of the *PfAG5* gene is likely responsible for early flowering in the R *P. fugax* population. As there was no clear trend in the expression of *IRP3* (homology to *FUL*), and as *AP1*/*FUL* gene (*FUL2*) may play a general role in regulating flowering time in monocots (Preston and Kellogg 2006), we assume that IRP3 may not play a major role in flowering regulation in *P. fugax*.

Recently, we identified another gene *PfMADS16* regulating early flowering and seed development in *P. fugax* (Zhou *et al*. 2020). The role of *PfAG5* identified in the current study is different to *PfMADS16* as the former is not involved in seed

development and interacts with different proteins. However, the two genes both play roles in flowering time regulation.

How has R *P. fugax* evolved higher expression of the flowering genes (such as *PfMADS16* and *PfAG5*) compared with the S population? Or how is early flowering trait correlated with herbicide resistance? According to Baucom (2019), 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 ACCase gene (Tang *et al*. 2014), and there has no evidence showing direct correlation of ACCase 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 latter can be examined by methylation analysis of the major 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 herbicide resistance becoming an increasing problem, 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.

Data availability statement

The identified *PfAG5* sequence in this paper has been deposited in the GenBank (accession number MK559453). Experimental materials are available upon request by qualified researchers to the corresponding author.

Conflicts of interest

The authors declare no conflicts of interest.

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