Overexpression of the Gm*GAL2* Gene Accelerates Flowering in *Arabidopsis*

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Abstract A soybean MADS box gene Gm*GAL2* (*Glycine max AGAMOUS Like 2*), a homolog of *AGL11/STK*, was investigated in transgenic *Arabidopsis* lines. Ectopic expression of Gm*GAL2* in *Arabidopsis* enhanced flowering, under both long-day and short-day conditions, by promoting expression of key flowering genes, *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*), and lowering expression of floral inhibiter *FLOWERING LOCUS C* (*FLC*). Moreover, frequency of silique pod set was also lower in transgenic compared to control *Arabidopsis* plants. RT-PCR results revealed that Gm*GAL2* was primarily expressed in the flowers and pods of soybean plants, Gm*GAL2* expressed higher in SD than LD in soybean.

Keywords Soybean (*Glycine max* [L.] Merr.) · Gm*GAL2* · MADS box · Flowering

Introduction

MADS box genes are found in both plants and animals and encode transcription factors that contain a highly conserved DNA-binding domain, which can regulate reproductive and vegetative developments (Alvarez-Buylla et al. 2000). In *Arabidopsis thaliana*, the MADS box gene family comprises more than 100 members, which can be grouped into five subfamilies, namely MIKC, Ma, Mb, Mg, and Md (Parenicova et al. 2003). The K box protein–protein interaction domain in MADS box proteins mediates the heterodimerization of MIKC-type MADS proteins that is necessary for the protein's function. MADS box genes control the identity of the apex meristem and floral organs, and the development of shoot, leaf, root, flower, and fruit (De Bodt et al. 2003; Foo et al. 2006; Irish 2003; Kater et al. 2006; Messenguy and Dubois 2003; Rijpkema et al. 2007; Robles and Pelaz 2005; Saedler and Huijser 1993). In addition, the MADS box proteins also regulate flowering time. For example, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) is an integrator of different flowering pathways and can promote flowering (Kim et al. 2001; Lee et al. 2004; Moon et al. 2003; Samach et al. 2000; Sheldon et al. 1999), whereas *FLOWERING LOCUS C* (*FLC*) acts as a repressor of flowering (Hepworth et al. 2002; Poduska et al. 2003; Rouse et al. 2002; Swarup et al. 1999). Some MADS box proteins have multiple functions. AP1, AGL24, and SVP act redundantly to control the identity of the floral meristem and to repress expression of class B, C, and E genes (Gregis et al. 2009). A MADS box gene AGL12 regulating root development and flowering was identified (Tapia-Lopez et al. 2008).

Seedstick/Agamous like 11 (STK/AGL11) is a key gene that controls ovule identity in *Arabidopsis*. In situ hybridizations revealed that *AGL11* RNA accumulated only in developing ovules and associated placental tissues; no *AGL11* RNA was detected in other floral organs during earlier or later stages of flower development (Rounsley et al. 1995). Ectopic expression of the *STK/AGL11* gene was sufficient to induce the transformation of sepals into

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carpeloid organs bearing ovules (Favaro et al. 2006; Pinyopich et al. 2003); it also resulted in the presence of curved rosette leaves and bracts, and the conversion of sepals into carpeloid organs that could develop mature ovules (Favaro et al. 2006). *STK* sequences are highly conserved among dicots and monocots; however, their functions are not always the same (Colombo et al. 1997; Lopez-Dee et al. 1999; Skipper et al. 2006). *FBP11*, an *AGL11* homolog in petunia, regulates ovule development; ectopic expression of *FBP11* induced the formation of ovules on the sepals and petals in petunia (Battaglia et al. 2006; Colombo et al. 1997, 1995). Ectopic expression of *LlMADS2*, an *AGL11* homolog from lily (*Lilium longiflorum*), caused the conversion of sepals and petals to carpel- and stamen-like structures in transgenic *Arabidopsis* plants (Tzeng et al. 2003). Interestingly, heterologous, ectopic expression of *AGL11* in *Arabidopsis* could not induce ectopic ovule formation. Ectopic expression of *OsMADS13*, an *AGL11* homolog from rice, failed to induce ectopic ovule formation as did *FBP11* in *Arabidopsis* (Favaro et al. 2006). These results indicate that *AGL11* might function in a species-dependent mode. *AGL11* also plays a role in flowering regulation. Overexpression of *LlMADS2* caused early flowering in lily (Tzeng et al. 2003). Such pleiotropic phenotypes are widely present in the plant kingdom. The blue light receptor, *Cryptochrome* (*CRY*) *2*, has been shown to be a regulator of flowering and growth of the hypocotyl (El-Din El-Assal et al. 2003; Guo et al. 1998), which also affects fruit length, ovule number per fruit, and percentage of unfertilized ovules (Guo et al. 1998).

The time to flower affects yield of soybean. As a short-day plant, flowering of soybean is sensitive to day length, which makes soybean an important model plant for photoperiod research (Zhang et al. 2008). We cloned and analyzed several MADS box genes from soybean by Rapid Amplification of cDNA ends (RACE). One of these soybean MADS box genes, Gm*GAL2* is a homolog of *AGL11/STK*. We found that soybean GAL2 affects flowering time in *Arabidopsis* when overexpressed.

Materials and Methods

Plant Growth

Seeds of soybean (*Glycine max* L. "KN 18") were sown in pots (diameter, 18 cm; height, 16 cm) with mixed field soil, turf soil, and vermiculite (2:2:1). Soybean plants were grown in a chamber with a controlled environment in short-day conditions (8 h/16 h, light/dark; 60 µmol m^{-2} s^{-1}) and long-day conditions (16 h/8 h, light/dark; 60 µmol m^{-2} s^{-1}) at a constant 25–28°C. *Arabidopsis* plants grew in long-day (16 h/8 h, light/dark) or short-day (8 h/16 h/, light/dark) conditions (60 µmol m^{-2} s^{-1}) at a constant 22°C. Data were

collected from more than 30 plants, and all experiments were repeated in triplicate.

RNA Preparation and Gene Cloning

For gene cloning RNA preparation, mixable shoot apical meristem of soybean on different stages (unifoliate, the first trifoliate, the second trifoliate, and the third trifoliate) were harvested in short-day conditions (8 h/16 h, light/dark). Different soybean organs were sampled at different stages in short-day conditions (8 h/16 h, light/dark), for example when each new leaf (unifoliate or trifoliate) expanded fully, for *GAL2* expression analysis. Ten-day-old *Arabidopsis* seedlings in long-day conditions (16 h/8 h, light/dark, light was provided from 8:00 am to 24:00 pm everyday) were harvested at 11:00 am. Soybean flowers and immature pods were sampled in long-day conditions (16 h/8 h, light/dark, light was provided from 8:00 am to 24:00 pm everyday) and short-day conditions (8 h/16 h, light/dark, light was provided from 8:00 am to 16:00 pm everyday). RNA was prepared with Trizol (Invitrogen) and reversed transcribed to cDNA with M-MLV RT (Fermentas).

We detected a set of ESTs that showed high sequence similarity to AGL11 (The Gene Index Project, http://compbio. dfci.harvard.edu/tgi/). Among them, AW705451 covered the 5′ terminal sequence of a candidate gene. Based on the sequences of AW705451, specific primers for RACE of the full length of candidate gene were designed: forward primer GAL2-F1 and reverse primer GAL2-R1 (Table 1). The reverse primer contained part of the adapter primer that was employed as a primer for reverse transcription, leading to higher efficiency. The resultant clones were sequenced to confirm their sequences.

RT-PCR and Quantitative PCR

RT-PCR was performed with the primers shown in Table 1. Quantitative PCR was carried out by ABI StepOne according to the manufacturer's instructions (ABI). Total cDNA (100 ng per reaction) was used as the template for RT-PCR. PCR products were analyzed using 1.2% agarose gel electrophoresis. All experiments were replicated in triplicate. The expression level of the *GmUBQ* gene was used as an internal control to normalize and calculate relative expression levels of genes tested using ImageJ software (http://rsb.info.nih.gov/ij/).

Constructing Expression Vectors and Plant Transformation

Construction of the expression vector was based on Gateway technology. The ORF of the *GAL2* gene was cloned into Entry clone pDONR201 by BP clonase (Invitrogen) and

Table 1 Primers employed in this study

Reaction	Name of primers	Sequence (5′ to 3′)
Reverse transcription	RTP	GACTCTGATGCTGACAATGACTTTTTTTTTTTTTTTTT
GAL2 cloning	GAL2-F1	TTCATTTCTTTGGATTAGAAATTTTTCC
	GAL2-R1	GACTCTGATGCTGACAATGACTTT
RT-PCR for GAL2	GAL2-F2	TGATGCCTTAAGCACACTGACT
	GAL2-R2	TGAGTGAGGGTAAACAGTTCCAC
Quantitative PCR for GAL2	GAL2-37F	AACACAACAAATCGGCAAGTGA
	GAL2-152R	CGGCTGGAGAAGACGATGAG
RT-PCR for AtCO	CO-F	CATTAACCATAACGCATACATTTCA
	CO-R	CTCCTCGGCTTCGATTTCTC
RT-PCR for AtFT	FT-F	GAACAACCTTTGGCAATGAGA
	FT-R	TCTTCCTCCGCAGCCACT
RT-PCR for AtSOC1	SOC1-F	CTAAACGTAAACTCTTGGGA
	SOC1-R	CAGAACTTGGGCTACTCTCT
RT-PCR for AtLFY	LFY-F	TCTCTCCCAAGAAGGGTTAT
	LFY-R	GTAGTGTCGCATTTTAGGCT
RT-PCR for AtAP1	AP1-F	GACGTCAATACAAACTGGTCGA
	AP1-R	GGAGATGGCTGATGAGAGAGC
RT-PCR for AtFLC	FLC-F	GGCGATAACCTGGTCAAGAT
	FLC-R	TAGTCACGGAGAGGGCAGTC
RT-PCR for AtUBQ10	AtUBQ-F	GATCTTTGCCGGAAAACAATTGGAGGATGG
	AtUBQ-R	CGACTTGTCATTAGAAAGAAAGAGATAACAGG
RT-PCR for GmUBQ	GmUBQ-F	CTCTGACAGGGAAGACCGTAAC
	GMUBQ-R	ACGAGACCGTGCATAGCAA

transferred to destination vector by LR clonase (Invitrogen). The resultant vector was a binary vector in which GAL2 was driven by the CaMV 35S promoter. It was transferred into *Arabidopsis* plants Ler with floral dipping approach mediated by *Agrobacterium* strain GV3101 90RK.

Phylogenetic Analysis

A phylogenetic analysis of protein sequences was carried out using the amino acid sequence alignment generated by CLUSTAL-W. A neighbor-joining tree was built using the software of MEGA version 3.1. Support for the tree was assessed using the bootstrap method with 1,000 bootstrap replicates. The numbers at each node represent the bootstrap support (percentage).

Results

Cloning the *GAL2* Gene

We used the sequence of AGL11 (At4g09960) as a query in a tBLASTn sequence search (http://www.ncbi.nlm.nih.gov/). We detected a set of ESTs that showed high sequence similarity with AGL11 (The Gene Index Project, http://compbio.dfci.harvard.edu/tgi/). Among them, AW705451 covered the 5′ terminal sequence of a candidate gene. Based on the sequence of AW705451, specific RACE primers for the full-length candidate gene were designed: forward primer GAL2-F1 and reverse primer GAL2-R1 (Table 1). The reverse primer was part of an adapter primer that was employed as a reverse transcription primer for higher efficiency. Using the RACE approach, we cloned a MADS box gene, *GAL2* (*Glycine max Agamous Like 2*). A BLAST analysis against the database of TAIR8 (http://www.arabidopsis.org/Blast) indicated that GAL2 is homologous to *Arabidopsis* AGL11 (Fig. 1a). Phylogenetic analysis (www.ncbi.nlm.nih.gov/blast) also suggests that *GAL2* belonged to the dicot family of *AGL11* (Fig. 1b). *GAL2* protein sequence had the highest similarity (95% identity) to *LjAGL11* and it had 75.2% identity with *AtAGL11*. GAL2 has relatively lower sequence similarity with the *AGL11* homologs in monocots. Like other MADS box proteins, *GAL2* has a MADS box (residues 3–57) and a K box (residues 74–177) at the N- terminus that are key to the MADS box functions. A bipartite nuclear localization signal (residues 9–26) was found within the MADS box region (Fig. 1a, http://myhits.isb-sib.ch/cgi-bin/motif_scan).

Fig. 1 Alignments of protein sequences of soybean *GAL2* and its homologs in *Arabidopsis thaliana* and *Lotus japonicus*. The *red box* denotes the MADS box, the *pink box* highlights the nuclear localization signal in the MADS box, and the *green box* shows the F box. The species abbreviations are: *At* (*Arabidopsis thaliana*); *CUM10* (*Cucumis sativus*); *EgMADS1* (*Elaeis guineensis*); *EgMADS1* (*Eustoma grandiflorum*); *FBP7* (*Petunia* x *hybrida*); *FBP11* (*Petunia* x *hybrida*); *GhMADS*-2 (*Gossypium hirsutum*); *HoMADS1* (*Hyacinthus orientalis*); *LjAGL11* (*Lotus japonicus*); *LlMADS1* (*Lilium longiflorum*); *LlMADS2* (*Lilium longiflorum*); *LcMADS1* (*Litchi chinensis*); *OsMADS13* (*Oryza sativa*); *SlAGL11* (*Solanum lycopersicum*); *VvMADS5* (*Vitis vinifera*); *ZAG2* (*Zea mays*); and *ZMM1* (*Zea mays*)
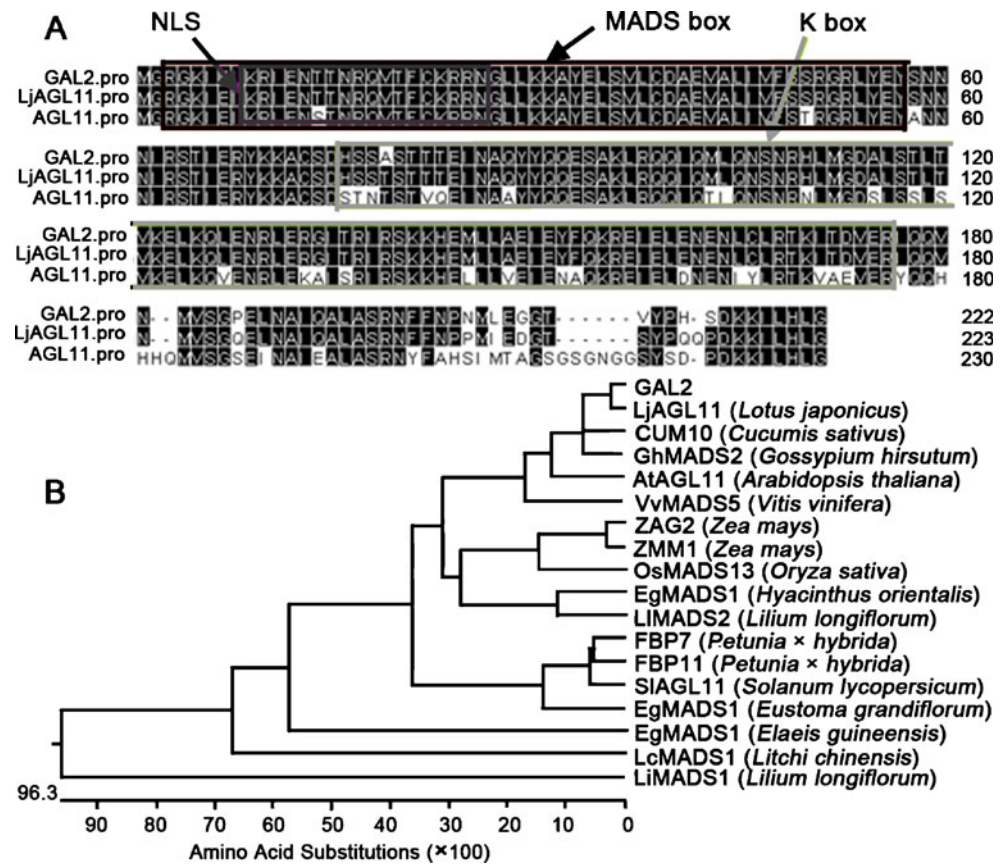
GAL2 Promotes Flowering in *Arabidopsis*

To study the function of *GAL2* in plant development, we overexpressed *GAL2* via the CaMV 35S promoter in *Arabidopsis* plants. The transgenic plants were analyzed by RT-PCR (Fig. 2e and f) and quantitative PCR (Fig. 2g) to confirm the presence and expression level of the transferred gene. Three transgenic lines showed *GAL2* expression. Transgenic plants expressing GAL2 flower earlier than the wild-type control; each line showed a different level of expression. It appears that the level of GAL2 expression correlates with the flowering time (Fig. 2d) and the total number of leaves (Fig. 2c). The acceleration of flowering in the transgenic plants expressing *GAL2* was more pronounced in short-day conditions than in long-day conditions (Fig. 2a and b). These results suggest that the activity of *GAL2* was partially dependent on photoperiod in *Arabidopsis*.

We next examined the level of expression of different flowering time genes in *GAL2*-overexpressing plants, including *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*), *CONSTANS* (*CO*), *APETALA1* (*AP1*), and *LEAFY* (*LFY*). Figure 3a shows that expressions of *FT*, *SOC1*, *CO*, *AP1*, and *LFY* increased in transgenic lines, while the expression of flowering suppressor, *FLC* was repressed (Fig. 3). Gm*GAL2* overexpression in *Arabidopsis* induces flowering and leads to elevated levels of *CO* and *FT* mRNA. Expression of *SOC1*, *AP1*, and *LFY* is also increased, presumably as a consequence of higher *FT* transcription, but it is also possible that overexpression in *Arabidopsis* of *STK* or Gm*GAL2* leads to artificial effects.

GAL2 Affects the Development of Different Organs in *Arabidopsis*

GAL2 also affected the development of different organs, just as *OsMADS13* did (Favaro et al. 2002a). In transgenic plants, all the leaves are small and curl upwards, and this phenotype is much stronger in long-day conditions than in short-day condition (Figs. 2a and b, 4b and c). Flowers are smaller and shorter than that in the wild-type (Fig. 4d and e). In particular, the petals and sepals are shorter and do not cover the pistil completely, so that the filaments are exposed, but the style is longer than the wild-type (Fig. 4d). The number of seeds in each silique is reduced because the mature silique is shorter (Fig. 4g) than that of wild-type plants (Fig. 4f). Moreover, overexpression of *GAL2* has some additional effects on plant development. As shown in Fig. 4g, the siliques in plants overexpressing *GAL2* have persistent petals. However, carpel- and stamen-like organs, and ovule-like structures, were not found on any flower organs in the transgenic flowers. It is conceivable that GAL2

Fig. 2 Overexpression of *GAL2* enhances flowering in *Arabidopsis*. Plants overexpressing *GAL2* flowered early in both long-day conditions (**a**) and short-day conditions (**b**), and had fewer leaves (**c**) and a shorter time of flowering from sowing (**d**). Transgenic plants were confirmed by RT-PCR (**e** and **f**; **e** shows the gel image and F shows figure plotted against the gel) and quantitative PCR (**g**). *UBQ* was used as the loading control for PCR
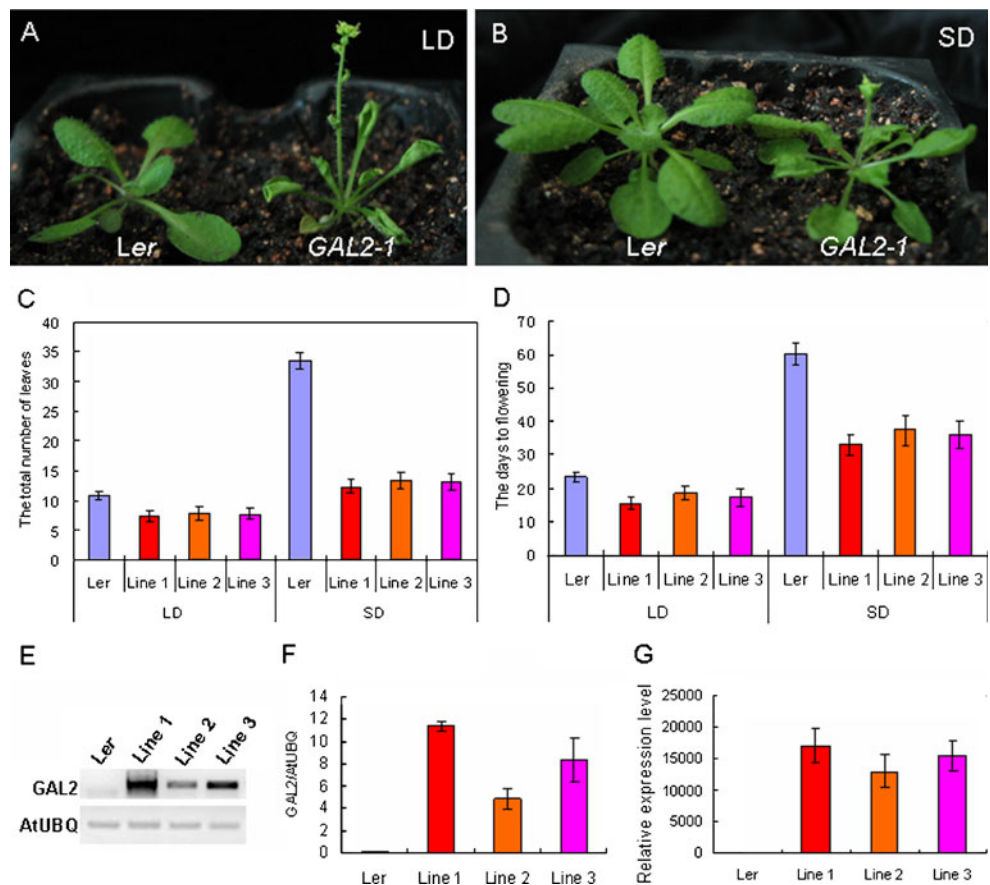
cloned from soybean might play additional roles in *Arabidopsis* development other than regulation of flowering time.

GAL2 is Mainly Expressed in Flowers and Pods in Soybean

The analysis of expression pattern of *GAL2* in soybean showed that *GAL2* is constitutively expressed in all organs and all developmental stages, but higher levels of expression were found in flowers and pods than in vegetative organs (Fig. 5). This observation is consistent with its hypothesized role in organ development in soybean.

GmGAL2 expressed higher in SD than LD in soybean.

The analysis of expression pattern of *GAL2* in soybean KN18 in short-day and long-day conditions showed Gm*GAL2* expressed higher in SD than LD in Soybean (Fig. 3b).
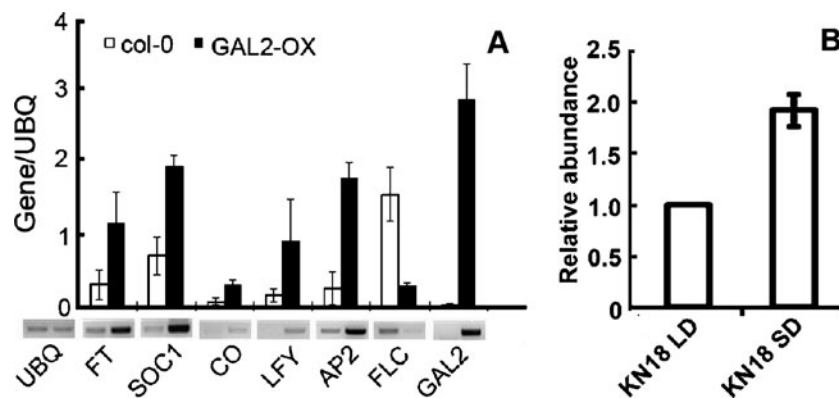
Fig. 3 The expression levels of flowering genes in transgenic *Arabidopsis* plants and the expression levels of GmGAL2 in soybean under long day and short day. **a**, Wild-type *Arabidopsis* seedlings and *GAL2*-overexpressing 10-day-old seedlings in long-day conditions (16 h/8 h, light/dark) were harvested 3 h after light provision. The expression levels of *Arabidopsis* flowering genes were tested by RT-PCR. **b**, GmGAL2 expressed higher in SD than LD in the flowers and mature pods of soybean KN18 plants
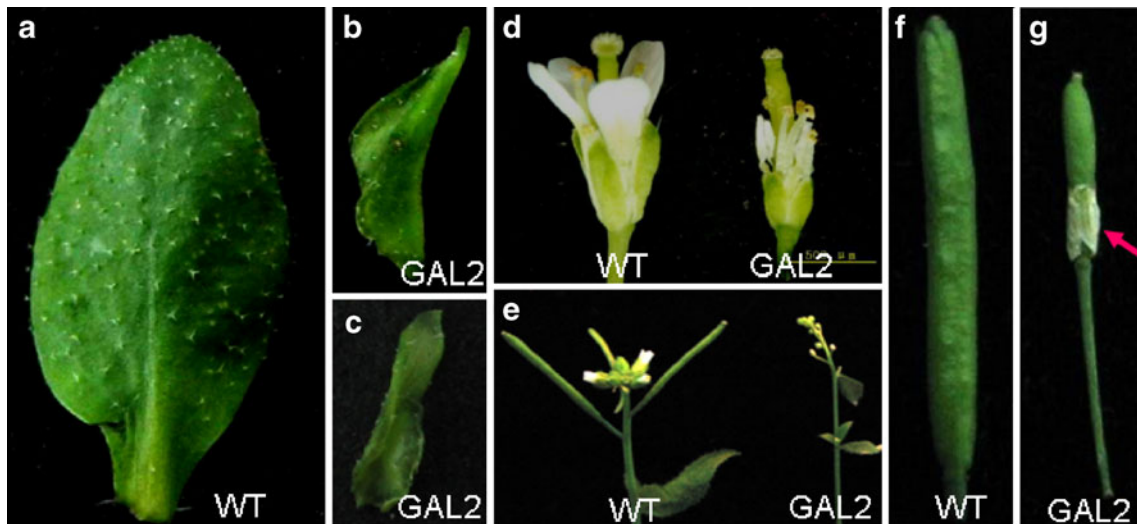
Fig. 4 The morphology of various organs in transgenic *Arabidopsis*. **a**, the leaf of the wild-type (*WT*); **b**, lateral view of a leaf of a transgenic plant; **c**, front view of a leaf of a transgenic plant; **d**, flowers of WT plants (*left*) and transgenic plants (*right*); **e**, The inflorescence of WT (*left*) and transgenic plants (*right*); **f**, the silique of a WT plant; **g**, the silique of a transgenic plant. An *arrow* shows the persistent petals on the silique

Discussion

We cloned a *STK/AGL11* homolog from soybean, *GAL2* (*Glycine max Agamous Like 2*). Its sequence shows high similarity to *AGL11* genes in many plants and it can be classed into the *AGL11* group of dicots. The sequence of *GAL2* is most similar to an *AGL11* protein of a Legume plant (*Lotus corniculatus*, with 95% identity), while it only has 75.2% similarity to *AGL11* of a Cruciferae plant (*A. thaliana*). The data suggests that *AGL11* might originate from the same ancestor but has evolved independently in dicots, monocots, long-day plants, and short-day plants. Thus, *AGL11* might have different functions between long-day plants and short-day plants, although they share some conserved functions. The sequence of *GAL2* shows typical characters of MADS box proteins, such as a highly conserved MADS box at the N-terminus, a nuclear localization signal in the MADS box, and a K box in the middle of the MADS box. These data suggest that *GAL2* is a MADS box gene and a putative *STK/AGL11* homolog in soybean.

Fig. 5 The expression levels in different organs or parts of soybean KN18. The development stages are: I, fully expanded unifoliolate; II, fully expanded first trifoliolate; III, fully expanded second trifoliolate; IV, fully expanded third trifoliolate; V, plants in anthesis; and VI, plants setting pods. *Upper panel* shows the gel images
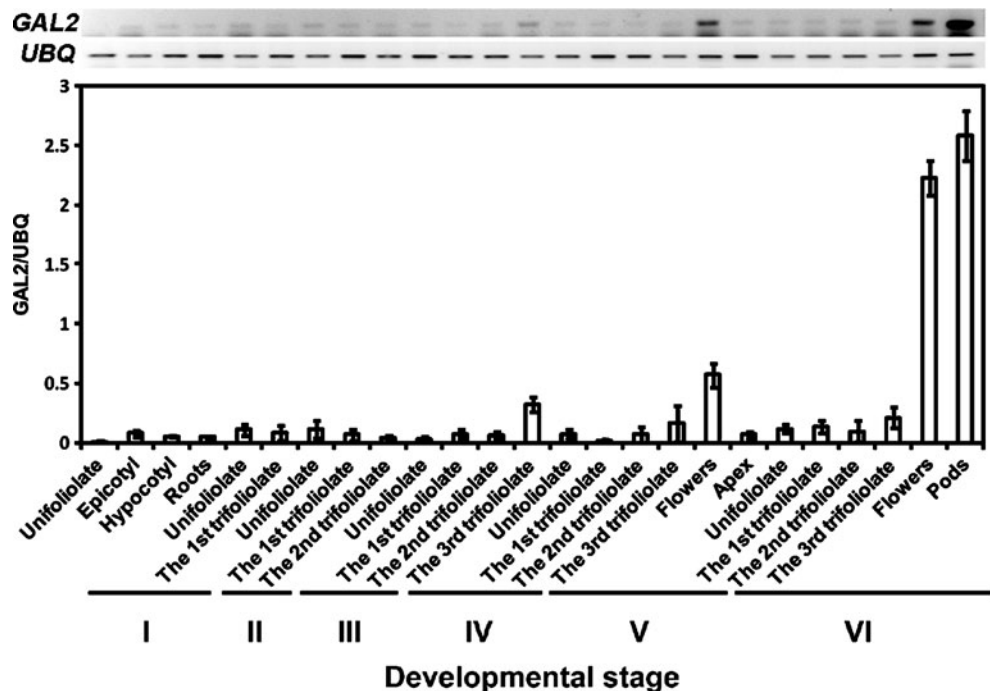
Like its homologs in other plants, ectopic expression of *GAL2* affects *Arabidopsis* development, but its behavior is different to that of any other *AGL11* homolog. As does its homolog in lily, overexpression of *GAL2* promotes flowering in *Arabidopsis* in both long-day conditions and short-day conditions; however, *GAL2* might not be involved in the regulation of circadian clock, because ox-Gm*GAL2* does not seem to affect the circadian clock in *Arabidopsis* (data not shown). *GAL2* does not affect the morphology of flower organs, including ovules and sepals, although it can cause smaller-sized flower organs. Soybean *GAL2* has additional functions in the development of flower organs. Flowers and siliques are smaller than those of wild-type plants, and persistent petals are obvious on the siliques (Fig. 4). In soybean, *GAL2* expresses at a higher level in flower organs and pods than in vegetative organs (Fig. 5). This suggests that *GAL2* is mainly involved in regulation of flowers and pods, in addition to its possible function in all the stages of soybean development.

From the expression patterns of flowering genes, *GAL2* participates in multiple pathways to enhance *Arabidopsis* flowering. In *GAL2* transgenic *Arabidopsis*, flowering activators, such as *CO*, *FT*, *SOC1*, *LFY,* and *AP1*, are up-regulated, while the flowering repressors (*FLC*) is down-regulated.

Despite the low level of expression in vegetative stages, *GAL2* might also function in the development of vegetative organs. The phenotype of the leaves (curly) of transgenic *Arabidopsis* supports that hypothesis (Fig. 4b, c). The effects of *AGL11* and its homologs on leaf development are dependent on species. The phenotype of curly leaves was observed in the bract leaves around flowers in *Arabidopsis* plants ectopically-expressing rice *OsMADS13* (Favaro et al. 2002). The expression of *AGL11* from *Arabidopsis* resulted in the presence of curved rosette leaves and bracts in transgenic *Arabidopsis* plants (Favaro et al. 2002). All the leaves have curly phenotype in plants overexpressing *GAL2* (Fig. 2a and b). Therefore, *GAL2*'s function in leaf development is not the same as that of *OsMADS13*, even though both rice and soybean are short-day plants. The effect of *GAL2* on leaf development might be in a photoperiod-dependent manner because the long-day condition enhances *GAL2* function (Fig. 2a and b).

In summary, *GAL2* is a homolog of *A. thaliana AGL11* in soybean. It has similar, but not identical, functions on plant development compared with its homolog in other plants.

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