# Control of Compound Leaf Development by *FLORICAULA/LEAFY* Ortholog *SINGLE LEAFLET1* in *Medicago truncatula*[1][C][W][OA]

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Molecular genetic studies suggest that *FLORICAULA (FLO)/LEAFY (LFY)* orthologs function to control compound leaf development in some legume species. However, loss-of-function mutations in the *FLO/LFY* orthologs result in reduction of leaf complexity to different degrees in *Pisum sativum* and *Lotus japonicus*. To further understand the role of *FLO/LFY* orthologs in compound leaf development in legumes, we studied compound leaf developmental processes and characterized a leaf development mutant, *single leaflet1 (sgl1)*, from the model legume *Medicago truncatula*. The *sgl1* mutants exhibited strong defects in compound leaf development; all adult leaves in *sgl1* mutants are simple due to failure in initiating lateral leaflet primordia. In addition, the *sgl1* mutants are also defective in floral development, producing inflorescence-like structures. Molecular cloning of *SGL1* revealed that it encodes the *M. truncatula* FLO/LFY ortholog. When properly expressed, *LFY* rescued both floral and compound leaf defects of *sgl1* mutants, indicating that LFY can functionally substitute SGL1 in compound leaf and floral organ development in *M. truncatula*. We show that *SGL1* and *LFY* differed in their promoter activities. Although the *SGL1* genomic sequence completely rescued floral defects of *lfy* mutants, it failed to alter the simple leaf structure of the *Arabidopsis thaliana* plants. Collectively, our data strongly suggest that initiation of lateral leaflet primordia required for compound leaf development involves regulatory processes mediated by the *SGL1* function in *M. truncatula*.

Leaves are determinate organs initiated from the periphery of the pluripotent shoot apical meristem (SAM). The class I *KNOTTED1*-like *(KNOX1)* homeobox genes are involved in acquisition and maintenance of the meristem activity of SAM (Long et al., 1996). Initiation of lateral organs such as leaves requires down-regulation of *KNOX1* genes at the incipient leaf primordia, and subsequent acquisition of determinacy in developing organs further requires continued suppression of *KNOX1* gene expression (Smith et al., 1992; Sinha et al., 1993; Jackson et al., 1994). Thus, development of lateral organs from the periphery of the SAM requires fine-tuned interactions of multiple regulators

in SAM, some maintaining the meristem fate, such as the *KNOX1* genes, and others promoting determinacy by suppressing expression of meristem genes, such as *ROUGH SHEATH2 (RS2)* and *ASYMMETRIC LEAVES1 (AS1)* in maize *(Zea mays)* and Arabidopsis *(Arabidopsis thaliana)*, respectively (Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000; Ori et al., 2000; Hay et al., 2006).

In compound-leafed species such as tomato *(Solanum lycopersicum)*, the expression of *KNOX1* genes is initially excluded from the incipient leaf primordia but subsequently reactivated in developing leaf primordia (Kim et al., 2003). Ectopic expression of *KNOX1* in tomato results in ramification of compound leaves, suggesting that *KNOX1* genes may play a role in compound leaf development (Hareven et al., 1996). Direct evidence that supports the role for *KNOX1* genes in compound leaf development comes from studies of *Cardamine hirsuta*, a small crucifer related to the simple-leafed model plant Arabidopsis. In transgenic *C. hirsuta* lines, in which expression of an endogenous *KNOX1* gene *SHOOTMERISTEMLESS* is down-regulated, leaflet initiation is reduced (Hay and Tsiantis, 2006). Thus, development of compound leaves involves reactivation of genes that promote indeterminacy in leaf primordia in compound-leafed species (Bharathan et al., 2002; Hay and Tsiantis, 2006; Jasinski et al., 2007; Ori et al., 2007).

The involvement of *KNOX1* genes in compound leaf development is evidenced in a number of compound-

leafed species including legumes (Bharathan et al., 2002; Hay and Tsiantis, 2006; Champagne et al., 2007). However, in some cases, the final leaf forms are not entirely correlated with the expression of *KNOX1* genes in leaf primordia, because some complex leaf primordia in which *KNOX1* genes are expressed may mature into simple leaves (Bharathan et al., 2002). In a large subclade of legumes, the inverted repeat-lacking clade (IRLC), including pea (*Pisum sativum*) and alfalfa (*Medicago sativa*), expression of *KNOX1* genes is excluded from the initiating leaf primordia (Gourlay et al., 2000; Hofer et al., 2001; Champagne et al., 2007). Thus, *KNOX1* genes are not likely correlated with compound leaf development in this group of legume plants (Hofer et al., 2001; Champagne et al., 2007).

Molecular genetic studies indicate that the legume orthologs of the floral meristem (FM) identity gene *FLORICAULA* (*FLO*) from snapdragon (*Antirrhinum majus*) and *LEAFY* (*LFY*) from Arabidopsis, and their coregulators *FIMBRIATA* (*FIM*) and *UNUSUAL FLORAL ORGAN* (*UFO*), respectively, play a role in compound leaf development in compound-leafed legumes (Hofer et al., 1997; Hofer and Ellis, 1998; DeMason and Schmidt, 2001; Taylor et al., 2001; Dong et al., 2005). The pea *unifoliata* (*uni*) and *stamina pistilloida* (*stp*) mutants exhibit inflorescence and floral defects that are similar to that of the snapdragon *flo* and *fim* mutants, and Arabidopsis *lfy* and *ufo* mutants, respectively, and in addition reduced compound leaf phenotypes (Hofer et al., 1997; Taylor et al., 2001). In *Lotus japonicus*, a legume species that is outside of the IRLC, *proliferating floral meristem* (*pfm*) mutants of the *FLO/LFY* ortholog also exhibit reduced compound leaf phenotypes (Dong et al., 2005). In transgenic soybean (*Glycine max*; outside of the IRLC) lines in which the endogenous *LFY* genes are down-regulated, the leaflet number is moderately reduced (Champagne et al., 2007). Taken together, these data support a significant role for the *FLO/LFY* and *STP/UFO* orthologs in compound leaf development in some legumes and a minor role in others.

Here, we describe compound leaf developmental processes in the model legume species *Medicago truncatula* and report characterization of a leaf development mutant, *single leaflet1* (*sgl1*) isolated from a tobacco (*Nicotiana tabacum*) *Tnt1* retrotransposon-tagged mutant population of *M. truncatula*. Our genetic analyses of four loss-of-function alleles of the *sgl1* mutant indicate that *SGL1* plays a key role in initiation of lateral leaflet primordia during an early stage of leaf development resulting in a complete conversion of compound leaves into simple leaves. We also show that *SGL1* controls the development of petioles along the proximodistal axis of compound leaves at a late developmental stage and FM identity during the reproductive phase of growth. Sequence analyses indicate that *SGL1* encodes a plant-specific protein related to the snapdragon FLO, Arabidopsis LFY, and pea UNI transcription factors. We discuss possible mechanisms by which *FLO/LFY* orthologs regulate compound leaf development in legumes.

## RESULTS

### Compound Leaf Development in *M. truncatula*

In pea, a legume species forming odd-pinnate compound leaves with distal tendrils, development of leaflet primordia is acropetal during early stages of leaf development. However, development of distal tendrils follows a basipetal pattern, giving rise to the unique compound leaf structure of pea plants (Hofer and Ellis, 1998). By contrast, the order of leaflet primordial development is basipetal in *L. japonicus* and other compound-leafed species (Hofer and Ellis, 1998; Gourlay et al., 2000; Luo et al., 2005). To facilitate characterization of leaf mutants in *M. truncatula*, we investigated leaf developmental processes with both visual and scanning electron microscopic (SEM) analyses. Similar to pea, *L. japonicus*, and other eudicot species, leaf development in *M. truncatula* is heteroblastic. However, the degree of heteroblasty is much simpler in *M. truncatula* than in other species, i.e. a single juvenile leaf with simple leaf morphology develops on the first node of a developing *M. truncatula* plant, and all other leaves that develop subsequently are in trifoliolate adult form, consisting of a pair of lateral (or proximal) leaflets and a terminal leaflet at the distal end of a petiole subtended by a pair of stipules (Fig. 1A). In *M. truncatula*, development of petioles was also heteroblastic, i.e. in 5-week-old plants, adult leaves developed on the third and fourth nodes had the longest petioles (Fig. 2H).

Morphological changes during compound leaf primordial development can be divided into 10 distinct stages in *M. truncatula*. At Stage 0 (S0), cells along the periphery of SAM were recruited and became an incipient leaf primordium, albeit no signs of outgrowth were visible at this stage (Fig. 1B). At S1, a common leaf primordium formed as a strip of cells outgrew along the periphery of SAM (Fig. 1B). At the subsequent S2, a pair of stipule primordia emerged as small bumps of cells that grew out of the proximal end of the common leaf primordium (Fig. 1C). At S3, a pair of lateral leaflet primordia emerged between the stipule and common leaf primordia (Fig. 1D). Because no additional primordia developed after this stage, the common leaf primordium differentiated into a terminal leaflet primordium. Subsequently, at S4, the stipule and lateral leaflet primordia were separated away from each other so that boundaries were established between the stipule and lateral leaflet primordia (Fig. 1E). Adaxial-abaxial specification of the terminal leaflet primordium was apparent at this stage, because trichomes initiated as spherical outgrowth from the abaxial surface of the terminal leaflet primordium, indicating that the adaxial-abaxial polarity first established in the terminal leaflet primordium. Following S4, the lateral and terminal leaflet primordia grew away from each other, and boundaries formed between them at S5 (Fig. 1F). At this stage, it was apparent that trichomes differentiated further as tubular trichomes elongated from the abaxial
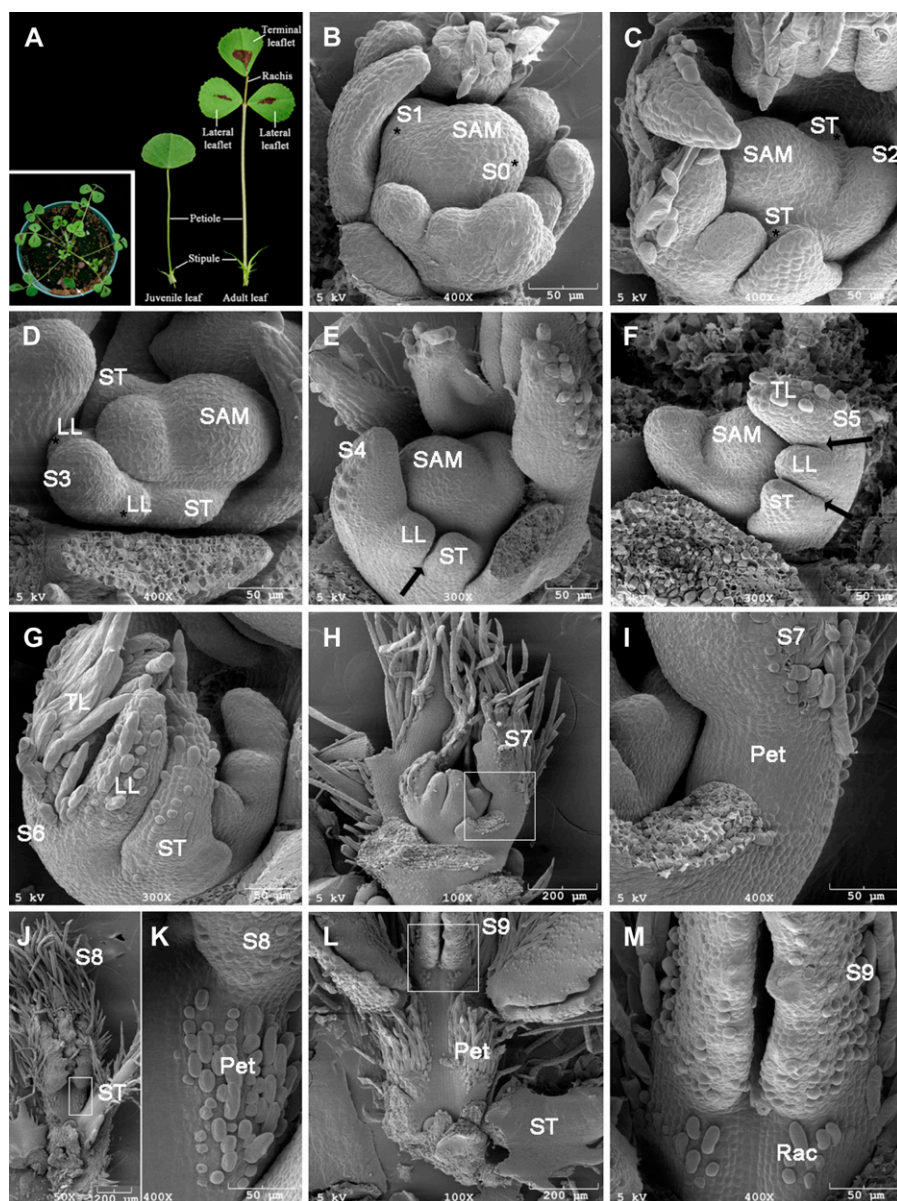
**Figure 1.** The ontogeny of compound leaf development in wild-type *M. truncatula*. A, Morphology of *M. truncatula* 'Jemalong' A17. Shown in inset were juvenile (left) and adult (right) leaves. B to M, SEM analysis of compound leaf development. B, Sites of incipient leaf primordia were specified at the periphery of the SAM at S0, albeit no morphological changes were visible at this stage. At S1, a common leaf primordium was initiated as a strip of cells outgrown along the periphery of SAM (asterisk). C, A pair of stipule primordia (ST) was initiated from the proximal end of the common leaf primordium at S2. D, At S3, a pair of lateral leaflet primordia (LL) emerged between the stipule and common leaf primordia. E, Boundaries (arrow) between the stipule and lateral leaflet primordia formed, and the common leaf primordium differentiated into a terminal leaflet primordium (TL) as indicated by development of trichomes from the abaxial surface at S4. F, At S5, boundaries (arrows) formed between the lateral and terminal leaflet primordia. G, While trichomes developed from the abaxial surface of both stipule and lateral leaflet primordia, the terminal leaflet primordium folded as a result of outgrowth of the abaxial surface at S6. H, At S7, a petiole primordium (Pet) formed between the stipule and lateral leaflet primordia. I, A close-up view of H. J, The lateral and terminal leaflet primordia folded due to outgrowth of the abaxial surface at S8. Trichomes developed from the adaxial surface of the petiole primordium at this stage. K, A close-up view of J. L, A rachis primordium (Rac) formed between the lateral and terminal leaflet primordia and trichomes developed from its adaxial surface at S9. M, A close-up view of L. Scale bars, 50 or 200 μm as indicated.

surface of the terminal leaflet primordium (Fig. 1F). At S6, trichomes developed from the abaxial surface of the stipule and lateral leaflet primordia. Furthermore, at this stage, the abaxial surface of the terminal leaflet primordium outgrew the adaxial surface such that the terminal leaflet primordium became folded (Fig. 1G). At the subsequent stage (S7), the region between stipule and lateral leaflet primordia expanded to become a petiole as a result of cell division and cell expansion (Fig.1, H and I). At S8, differentiation of petioles was apparent as trichomes initiated from the adaxial surface of petioles. Furthermore, at this stage, the region between lateral and terminal leaflet primordia expanded to form a rachis (Fig. 1, J–M). And, at the last stage (S9), the proximal regions of lateral and terminal leaflet primordia expanded to form petiolules (Fig. 1, L and M; data not shown).

## Isolation and Characterization of *M. truncatula* *sgl1* Mutants

We screened and isolated a leaf development mutant with four alleles from a *M. truncatula* mutant collection generated by tobacco *Tnt1* retrotransposon insertion mutagenesis (d'Erfurth et al., 2003; Tadege et al., 2005; Tadege et al., 2008). These mutants were named *sgl1-1* to *sgl1-4*, because all adult leaves are simple in these mutants, resembling the first leaf (juvenile leaf) developed in the wild-type plants (Figs. 1A and 2, A–C). Flowers developed in *sgl1* mutants were abnormal and infertile, lacking petals and stamens and producing many more flowers within flowers with cauliflower-like morphology (Figs. 2G and 3, A–C). Because of their infertility, the *sgl1* mutants were maintained as heterozygotes. Progenies from self-pollination of heterozy-

**Figure 2.** Phenotypes of *M. truncatula sgl1* mutants. Three-week-old wild-type *M. truncatula* (ecotype R108; A) and *sgl1-1* mutant (B) exhibited compound and simple leaf forms, respectively. C, Close-up views of adult leaves of wild type R108 (left) and *sgl1-1* mutant (right). In S2 and S4 (D), S3 and S5 (E), and S4 and S6 (F) leaf primordia of *sgl1-1* mutant, lateral leaflet primordia did not form at the proximal end of common leaf primordia. G, Morphology of a mature *sgl1-1* mutant plant, exhibiting simple leaf and floral homeotic phenotypes. H, Petiole length was significantly reduced in *sgl1-1* mutant plants compared to the wild-type R108 plants, but no significant differences in petiole length were found in newly developed leaves of wild-type and *sgl1-1* mutant (node 10). SL, Single leaflet; ST, stipule. Scale bars, 10 mm (A–C); 50 $\mu$m; or 100 $\mu$m as indicated (D–F). [See online article for color version of this figure.]
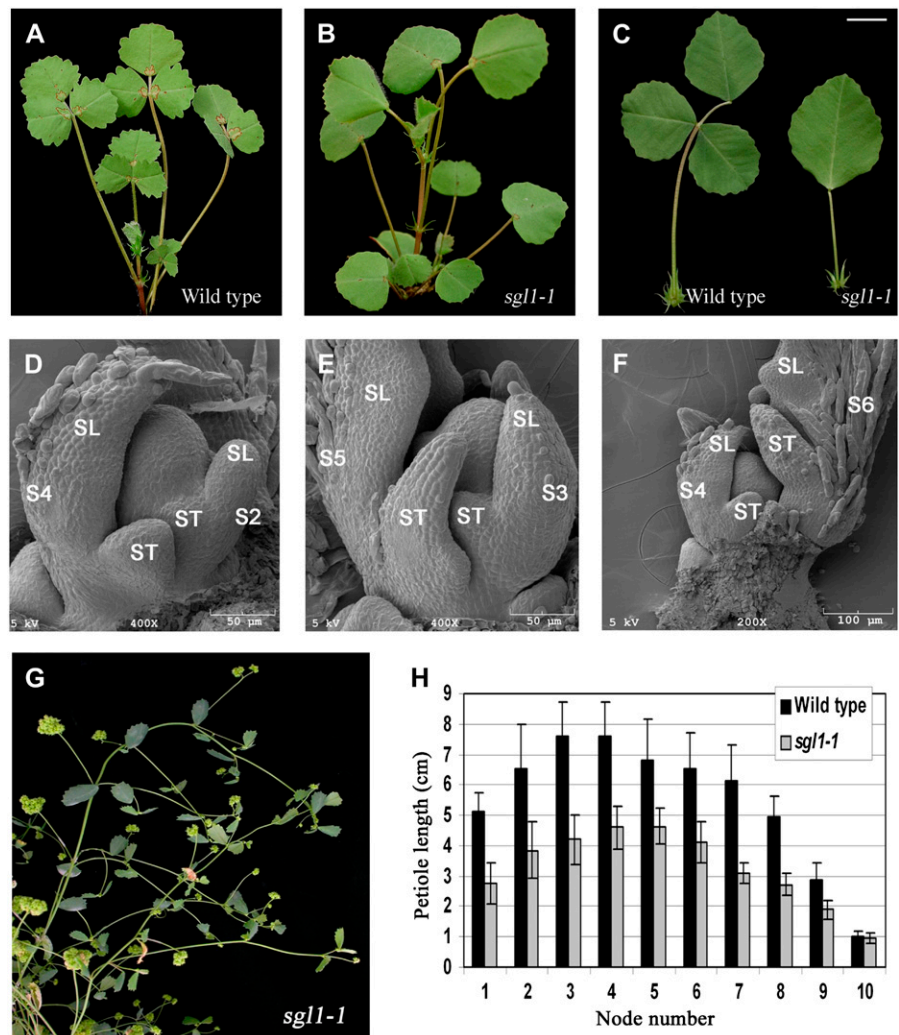


gous lines segregated wild-type-like and mutant plants in a 3:1 ratio, suggesting that the mutant phenotype was linked to a single recessive locus (Supplemental Table S1).

To characterize leaf development defects in *sgl1* mutants, SEM analyses of leaf development were carried out. These analyses indicated that in *sgl1* mutants, leaf development was initially normal until it reached S3, in which the pair of lateral leaflet primordia failed to initiate between the stipule and common leaf primordia (Fig. 2, D–F). All four alleles of the *sgl1* mutant exhibited identical defects. The defect in the initiation of lateral leaflet primordia was persistent throughout subsequent developmental stages, resulting in the formation of simple adult leaves in *sgl1* mutants (Fig. 2, A–G).

We also examined development of petioles in *sgl1* mutants and found that the length of petioles on newly developed leaves at node 10 was not significantly different between *sgl1* mutants and the wild-type plants (Fig. 2H). However, petioles on older leaves were significantly shorter in the *sgl1* mutants than in the wild-type plants (Fig. 2H), indicating that *SGL1* also plays a role in petiole development.

*M. truncatula* flowers have pentamerous organs in the outermost four whorls (sepals, petals, and outer and inner stamens) and a single carpel in the center. The development of *M. truncatula* flowers has been previously described (Benlloch et al., 2003). To characterize floral defects of *sgl1* mutants, we compared floral development of *sgl1* mutants with that of wild-type plants. Consistent with previous studies, we observed that during the reproductive phase of growth, one to three FMs were produced from a secondary inflorescence meristem (I$_2$) in wild-type plants (Fig. 3, A and D). From FMs, floral organ primordia were unidirectionally initiated along the abaxial-adaxial axis in a bilateral symmetric pattern (Fig. 3, E–G). At late S3 development, the abaxial and two lateral sepal primordia (Sl) were initiated from the outermost layer (Fig. 3E). At the subsequent stage, the abaxial common primordium and the central carpel primordium were initiated, followed by the initiation of two lateral common primordia and the adaxial common primordium (Fig. 3, E
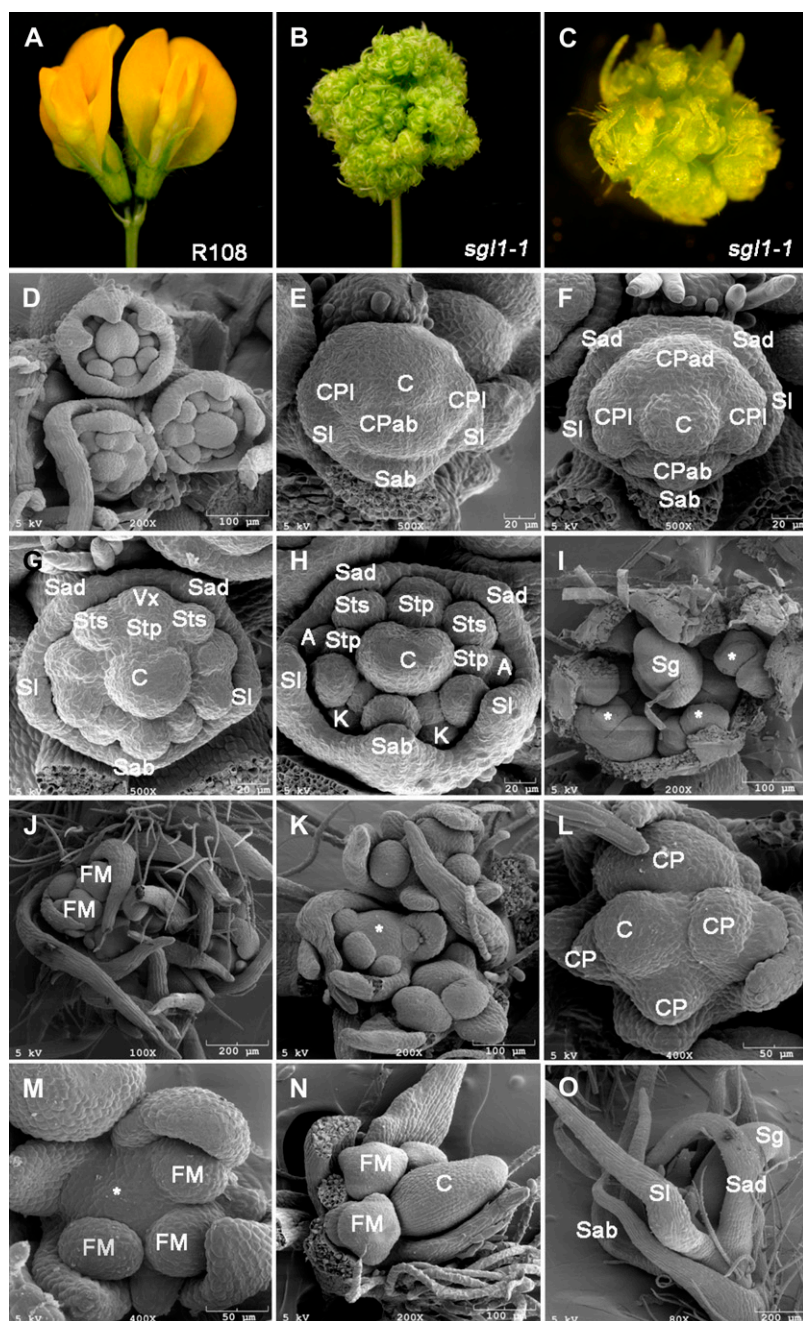
**Figure 3.** Flower phenotypes of *M. truncatula sgl1* mutants. Two mature flowers developed on a single spike in wild type (R108). The bilateral symmetry along the dorsal-ventral axis was shown. B, Defective flowers developed on a single spike in *sgl1-1* mutant. C, A close-up view of a single flower of the *sgl1-1* mutant. D to I, Flower development in wild type (R108). D, Three FMs were initiated from $I_2$. E, In S3, the abaxial (Sab) and two lateral (Sl) sepal primordia were initiated. F, In S4, two adaxial sepal primordia (Sad) were initiated. The carpel primordium (C) and the abaxial (CPab) and two lateral (CPl) common primordia formed. G, In S5, four common primordia differentiated into petal and stamen primordia along the abaxial to adaxial axis. Subsequently, the adaxial common primordia produced one inner antepetal (Stp) and two outer antesepal (Sts) stamens and the standard primordium (Vexillum or Vx). H, At subsequent stages, all floral organ primordia formed. A, Alae petals; K, keel petals. I, Stamen primordia differentiated anthers at S8 (asterisk). Sg, Stigma. J to O, Flower development in *sgl1* mutants. J, Multiple FM developed from a single $I_2$ inflorescence meristem in *sgl1-1* mutant. K, A close-up view of an $I_2$ with multiple FM. L and M, An S5 FM in *sgl1-1* mutant initiated three to six common primordia (CP) between sepal (S) and carpel (C) primordia. The carpel primordium was occasionally missing from the center of the FMs (asterisks in K and M). M, Secondary FM were initiated from the second whorl of FMs in *sgl1-1* mutant. N, An S7 FM in *sgl1-1* mutant with its carpel primordium (C) started to fold. Secondary FM started to differentiate. O, Secondary floral-like meristems developed between the carpel and sepal primordia and gave rise to proliferating structures with elongated sepals (S). Scale bars as indicated. [See online article for color version of this figure.]

and F). Between S4 and S5, the four common primordia in the second whorl differentiated to give rise to petal and stamen primordia along the abaxial-adaxial axis (Fig. 3G; alae petal, keel petal, inner antepetal stamen, and outer antesepal stamen). At late S5, all floral primordia were developed (Fig. 3H). Later, at S7, the stigma of the central carpel was folded and the stamen primordia differentiated filaments and anther locules (Fig. 3I; asterisk). By contrast, in *sgl1* mutants, three to five incomplete FMs were initiated from $I_2$ (Fig. 3, B, C, J, and K). Furthermore, even though sepals and the central carpel occasionally formed (Fig. 3, K, L, and O), petals and stamens were missing in *sgl1* mutants (Figs.

3, N and O). Instead, the common primordia developed from the second whorl formed three to five incomplete FMs, which gave rise to defective flowers of similar inflorescence-like morphology (Fig. 3, J–N).

## Molecular Cloning of the *SGL1* Gene

The floral homeotic defects of the *sgl1* mutants resemble that of *uni* and *stp* mutants. PCR amplification of *M. truncatula STP* genomic sequence from *sgl1* mutants and wild-type plants yielded identical products, indicating that the *M. truncatula STP* gene is not interrupted in the *sgl1* mutants (data not shown). On

the contrary, PCR amplification of *M. truncatula FLO/ LFY/UNI* genomic sequence from the *sgl1* mutants and the wild-type plants exhibited a difference of 5.3 kb, suggesting that each of the four *sgl1* alleles carried a single *Tnt1* insert (Fig. 4A; data not shown).

Flanking sequence analyses indicated that *Tnt1* was inserted in the corresponding *SGL1* gene in the first exon at positions 198, 314, and 333 bp, and in the third exon at the position 831 bp downstream from the translation initiation codon ATG in *sgl1-1* to *sgl1-4* alleles, respectively (Fig. 4B). Furthermore, the orientation of *Tnt1* in *sgl1-1* and *sgl1-4* was opposite from that in *sgl1-2* and *sgl1-3* alleles (Fig. 4B).

## Cosegregation and Genetic Complementation Analysis

All four *sgl1* alleles were infertile. Segregation analysis of an $F_2$ population of the *sgl1-1* allele indicated that 34 out of a total of 139 individuals were homozygous for *Tnt1* insertion and exhibited both simple leaf and floral homeotic defects, suggesting that *Tnt1* insertion in the corresponding *SGL1* gene cosegregated with the mutant phenotype (Supplemental Table S1).

To confirm that we have cloned the *SGL1* gene, we carried out a genetic complementation test in *sgl1-1* mutant using *Agrobacterium tumefaciens*-mediated stable transformation with a wild-type *SGL1* genomic sequence. Phenotypic analysis of transgenic plants indicated that three lines were completely rescued,

exhibiting both wild-type-like compound leaves and flowers (Figs. 4C; data not shown). The other two lines were only rescued for the compound leaf phenotype (data not shown), the floral phenotype of which was only partially rescued in one line but not at all in the other line (Fig. 4D).

We examined the expression level of the introduced *SGL1* gene in these stable transgenic lines using reverse transcription (RT)-PCR. The results indicated that the introduced *SGL1* gene was expressed in all five transgenic lines (Fig. 4E). However, the expression level was much higher in lines that exhibited fully and partially rescued flowers than in the line with nonrescued flowers (Fig. 4E).

Thus, the interruption of the same gene in four independent lines together with the genetic complementation data unambiguously confirmed that the *SGL1* gene is the *M. truncatula FLO/LFY/UNI* ortholog.

Comparison of the genomic and full-length complementary DNA (cDNA) sequences of *SGL1* indicate that *SGL1* consists of three exons and two introns (Fig. 4B). Furthermore, the intron and exon structure of *SGL1* was very similar to that of *FLO*, *LFY*, and *UNI*. Phylogenetic analysis of selected FLO/LFY orthologs from a diverse group of species placed SGL1 in close proximity to UNI from pea and PFM from *L. japonicus* (Fig. 4F). Together, they formed a cluster that is distantly related to FLO from snapdragon (Coen et al., 1990), LFY from Arabidopsis (Weigel et al., 1992), and FLO/
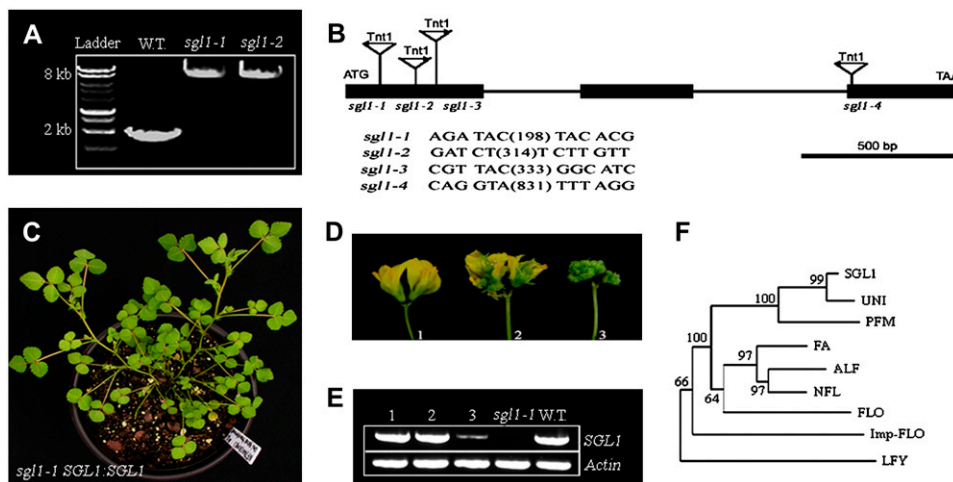


**Figure 4.** Molecular cloning of *SGL1* gene and functional complementation of *sgl1-1* mutant. A, PCR amplification of *SGL1* gene from wild-type *M. truncatula* (ecotype R108), *sgl1-1*, and *sgl1-2* mutants. A single insertion of tobacco *Tnt1* retrotransposon was detected as a shift in molecular weights of the amplified *SGL1* gene from each *sgl1* mutant allele. B, The intron and exon structure of *SGL1* and positions and orientation of *Tnt1* insertions in *sgl1* mutants. *Tnt1* was inserted in the first exon at positions 198 bp, 314 bp, and 333 bp, and in the third exon at the position 831 bp downstream from the translation initiation codon of *SGL1* in *sgl1-1* to *sgl1-4* mutants, respectively. C, A representative *sgl1-1* transgenic line transformed with the *SGL1* genomic sequence (*SGL1*:*SGL1*) exhibited completely rescued wild type-like leaves. D, Representative flowers of three independent *sgl1 SGL1*:*SGL1* transgenic lines, showing completely rescued (line 1), partially rescued (line 2), and nonrescued (line 3) flowers, albeit leaves from all three lines were rescued. E, RT-PCR analysis of *SGL1* expression level in developing flowers of three independent *sgl1 SGL1*:*SGL1* transgenic lines (lanes 1–3) compared with that of the *sgl1-1* mutant (lane 4) and wild type (lane 5). An *Actin* gene was used as an internal control (bottom). F, Phylogenetic analysis of SGL1 and its putative orthologs: FLO of snapdragon, LFY of Arabidopsis, NFL of tobacco, UNI of pea, ALF of petunia, Imp-FLO of impatiens, FA of tomato, and PFM of *L. japonicus*. Bootstrap supports above 50% from 1,000 replicates were shown. [See online article for color version of this figure.]

LFY orthologs from other species: Imp-FLO from impatiens (*Impatiens balsamina*; Pouteau et al., 1998), NFL1 and NFL2 from tobacco (Kelly et al., 1995), ABBERANT LEAF AND FLOWER (ALF) from petunia (*Petunia hybrida*; Souer et al., 1998), and FALSIFLORA (FA) from tomato (Molinero-Rosales et al., 1999).

### Tissue-Specific Expression of *SGL1*

RNA in situ hybridization data revealed that *SGL1* was expressed in the SAM and emerging leaf primordia (Fig. 5A). The highest expression was detected in the distal region of leaf primordia (Fig. 5A). During the reproductive phase of growth, transcripts of *SGL1* were detected in the $I_2$ (Fig. 5A). *SGL1* transcripts were also detected in the peripheral region of FMs (data not shown). During S7 of floral development (Benlloch et al., 2003), *SGL1* transcripts were detected in petal primordia (Fig. 5B).

To gain a better spatial and temporal resolution, we fused the *SGL1* promoter to the *Escherichia coli uidA* gene encoding GUS and introduced the resulting *SGL1:GUS* construct into *M. truncatula* wild-type plants (R108 ecotype). Out of four independent transgenic lines obtained, three showed strong and consistent GUS staining patterns, and the remaining one did not show any detectable GUS activity. We found that GUS staining from the GUS positive lines was restricted to the SAM and emerging leaf anlagen during early stages of leaf development (Fig. 5D, inset), consistent with our RNA in situ hybridization data. Interestingly, we observed that strong GUS staining was in the entire young leaflets (Fig. 5D, inset). In older leaves, GUS staining

was detected at the proximal region of leaflets and regions in rachis where leaflets were attached (Figs. 5, D–F). GUS staining in leaves was gradually reduced and eventually disappeared when leaves aged (Fig. 5G). In petioles and rachis, GUS staining was mainly detected in the vascular tissues (Figs. 5, D–F).

### Functional Conservation between LFY and SGL1

Previous studies have shown that *FLO* or *LFY* encodes a plant-specific transcription factor that controls FM identity and plant architecture (Coen et al., 1990; Weigel et al., 1992; Yoon and Baum, 2004). The role of *FLO/LFY* and their orthologs from angiosperm species in FM identity control is highly conserved (Maizel et al., 2005). Other studies suggest that mutations in *LFY* coding and/or 5′ upstream sequences are involved in driving morphological variations in closely related species during evolution (Yoon and Baum, 2004). Thus, it is not clear whether development of different leaf forms between Arabidopsis and *M. truncatula* is attributed to differences in sequences between *LFY* and *SGL1*. To test this, we carried out reciprocal genetic complementation experiments in Arabidopsis *lfy* and *M. truncatula sgl1* mutants. First, we transformed homozygous *sgl1* mutants with a full-length *LFY* cDNA driven by the native *SGL1* promoter (*SGL1:LFY*). Out of six stable transgenic lines obtained, all showed rescued phenotypes: wild-type-like compound leaves and flowers (Fig. 6, A and B, E–N). RT-PCR analyses indicated that the introduced *LFY* gene was expressed in these transgenic lines (Figs. 6, C and D). Furthermore, development of floral organs was also rescued in the transgenic lines (Fig. 6, E–N). Although *LFY* driven by
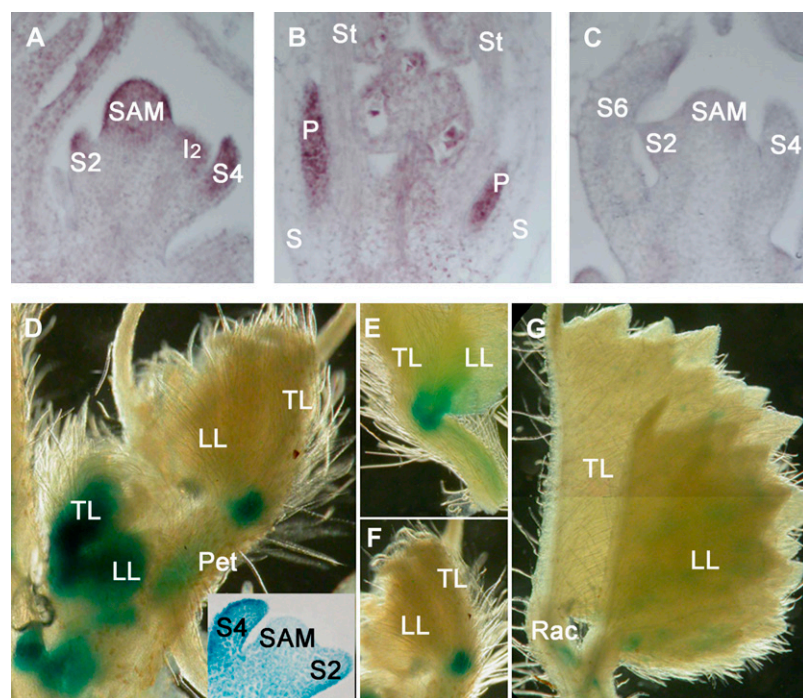


**Figure 5.** Expression pattern of *SGL1* gene. A to C, RNA in situ hybridization analysis of *SGL1* gene expression. A, *SGL1* gene expression was detected in SAM, developing leaf primordia (S2 and S4), and $I_2$. B, *SGL1* gene expression was detected in developing floral organs in S7 flowers of wild-type plants. P, Petal; S, sepal; St; stamen. C, *SGL1* sense probes were used as a negative control, no hybridization signal was detected in SAM and leaf primordia (S2, S4, and S6). D to G, *SGL1:GUS* histochemical staining pattern. D, GUS staining was restricted to the SAM (inset), developing leaf primordia at early stages (inset), vascular tissues of petioles, and the basal regions of leaflets at late stages. E and F, Close-up views of GUS staining patterns at the basal region of leaflets. G, GUS staining in mature leaves.
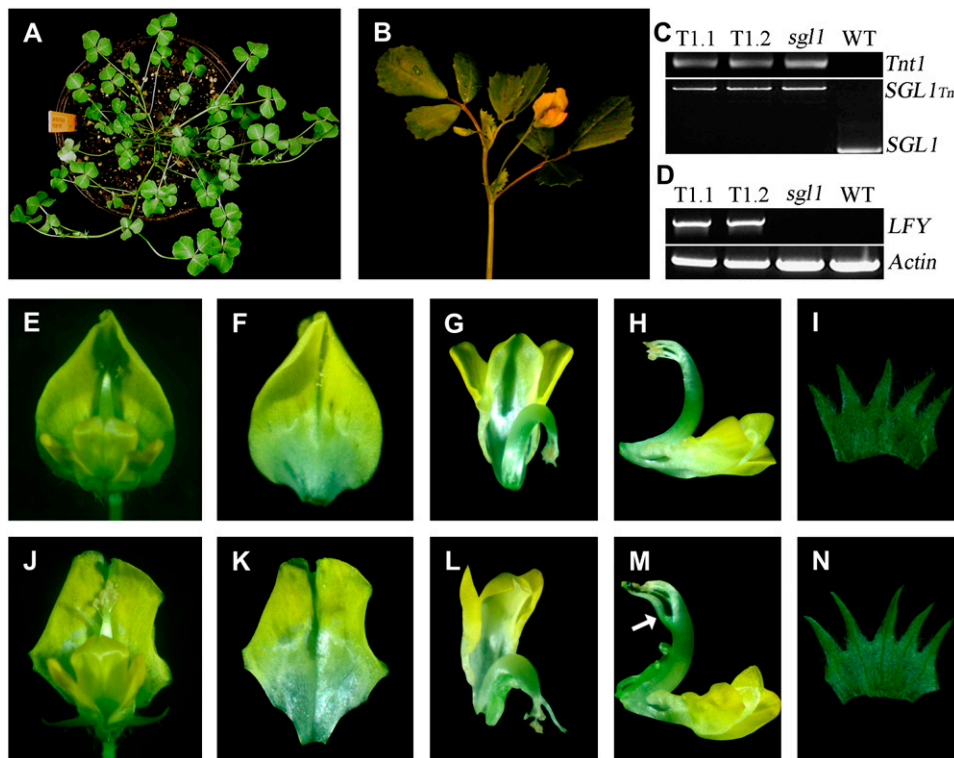
**Figure 6.** Genetic complementation of *sgl1* mutants by *LFY*. A, Homozygous *sgl1-1* mutant plants transformed with *SGL1:LFY*, all adult leaves exhibiting wild-type compound leaf morphology. B, Wild-type-like flowers were developed from secondary inflorescence from axils of compound leaves of *sgl1 SGL1:LFY* transgenic lines. C, PCR-based genotyping of *sgl1 SGL1:LFY* transgenic lines indicated that three independent transgenic lines were homozygous for *Tnt1* insertion in *SGL1* gene (lanes 1–3) in contrast to the wild-type *M. truncatula* (ecotype R108), where no *Tnt1* insertion was detected in *SGL1* gene (lane 4). Top segment for detecting *Tnt1* inserts; bottom for detecting *Tnt1* insertion in *SGL1*. D, RT-PCR analysis of expression of *LFY* gene in *sgl1 SGL1:LFY* transgenic lines. Shown were *LFY* expression in two independent *sgl1 SGL1:LFY* transgenic lines (lanes 1 and 2), *sgl1-1* mutant (lane 3), and wild-type *M. truncatula* (lane 4). Expression of an *Actin* gene was used as an internal loading control (bottom). E, A typical mature flower of wild-type *M. truncatula* (ecotype R108), exhibiting bilateral symmetric zygomorphic morphology. F to I, Floral organs of wild-type *M. truncatula* were dissected. Shown were a top view of vexillum (F), top (G), and side (H) views of keel and alae, the central carpel enclosed by the staminal tube, and a top view of dissected sepals (I). J, A representative mature flower of *sgl1 SGL1:LFY* transgenic lines, exhibiting wild-type-like morphology. K to N, Floral organs of *sgl1 SGL1:LFY* transgenic lines were dissected. Shown were a top view of vexillum (K), top (L) and side (M) views of keel and alae, the central carpel, and a top view of dissected sepals (N).

the native *SGL1* promoter rescued both leaf and floral defects of *sgl1-1* mutant, we observed some minor developmental defects in the transgenic lines, i.e. the central carpel appeared to elongate slightly faster than the staminal tube such that it often curled and protruded out of the staminal tube in the transgenic lines (Fig. 6, E–N, arrow).

Second, homozygous *lfy* mutant plants were transformed with the full-length *SGL1* cDNA driven by the *LFY* promoter. Sixteen out of 17 transgenic lines expressed the *SGL1* gene and rescued the *lfy* floral defects (Figs. 7, A–C, E and F; Supplemental Table S2). The remaining line did not express the *SGL1* gene to a detectable level and did not rescue the *lfy* phenotype (data not shown). However, both rosette and cauline leaves of *lfy LFY:SGL1* transgenic lines remained to be simple (Fig. 7C; data not shown). These data indicate that *SGL1* and *LFY* are also functionally conserved in control of FM identity and that expression of *SGL1*

driven by the *LFY* promoter was not sufficient to alter the simple leaf structure of the Arabidopsis plants.

### *M. truncatula SGL1* Genomic Sequence Functionally Rescued *lfy* Floral Defects But Failed to Alter the Simple Leaf Structure of the Arabidopsis Plants

Even though *LFY* was expressed at the periphery of vegetative SAM, its expression was largely excluded from developing leaves and pedicels (Weigel et al., 1992; Yoon and Baum, 2004; Fig. 7, H–L). By contrast, the *M. truncatula SGL1* gene was expressed in the entire SAM, including the central rib region, the initiating leaflet primordia, and developing leaflets (Fig. 5, A, D–G). In transgenic Arabidopsis lines, the introduced *SGL1:GUS* reporter gene was also expressed in vascular bundles and at margins of developing leaves, and in sepals, pedicels, inflorescence stems, and styles of flowers (Fig. 7, M–Q).
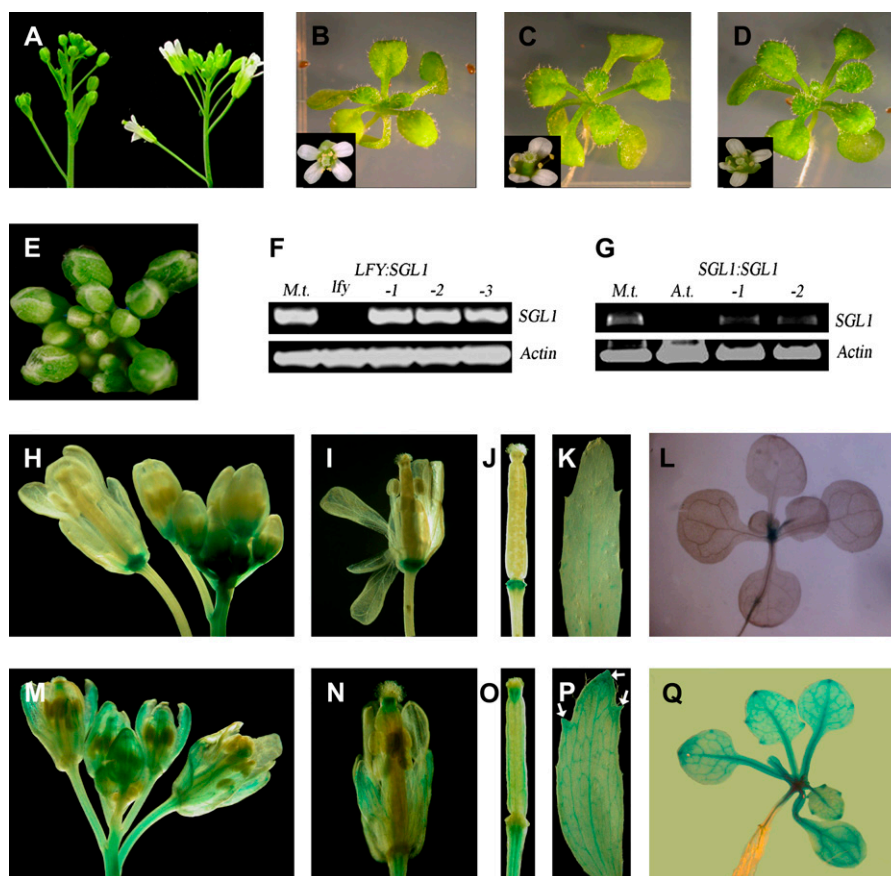
**Figure 7.** Genetic complementation of *lfy* mutants by *SGL1* and comparison of expression patterns of *LFY:GUS* and *SGL1:GUS* in Arabidopsis. A, Inflorescence of homozygous *lfy* (left) and *lfy* transformed with *LFY:SGL1* (right). B to D, Two-week-old wild-type (B), *lfy LFY:SGL1* (C), and *lfy SGL1:SGL1* (D) seedlings; insets, mature flowers. E, Morphology of *lfy* mutant flowers. F, RT-PCR analysis of *SGL1* expression in wild-type *M. truncatula* (lane 1), *lfy* (lane 2), and three independent transgenic *lfy* lines transformed with *LFY:SGL1* (lanes 3–5). G, RT-PCR analysis of *SGL1* expression in *M. truncatula* (lane 1), Arabidopsis (lane 2), and two independent transgenic *lfy* lines transformed with the *SGL1* genomic sequence (*SGL1:SGL1*; lanes 3 and 4). An *Actin* gene was used as an internal loading control (F and G; bottom). H and L, Histochemical staining of *LFY:GUS* in mature and young seedlings. Strong GUS staining was restricted to the base of flowers, vascular tissues of inflorescence stems, and the shoot apex but not of pedicels and leaves. M to Q, Histochemical staining of *SGL1:GUS* in mature and young seedlings. Strong GUS staining was detected in style, but not at the base of flowers, and in vascular tissues of inflorescence stems, pedicels, sepals, carpel, cauline, and rosette leaves, and the shoot apex. In cauline and rosette leaves, a high level of GUS staining was localized to the distal margins (arrows).

To address the question whether differences in the expression patterns between *LFY* and *SGL1* underlie development of different forms of leaves in Arabidopsis and *M. truncatula*, we generated homozygous *lfy* transgenic lines transformed with the *SGL1* genomic sequence. Out of 17 transgenic lines obtained, all properly expressed the *SGL1* gene and rescued the *lfy* floral defects (Fig. 7, D and G; Supplemental Table S2), indicating that the *SGL1* genomic sequence is sufficient to rescue the *lfy* floral defects, despite differences in the promoter activities between *SGL1* and *LFY*. However, both rosette and cauline leaves in transgenic *lfy SGL1:SGL1* lines remained to be simple (Fig. 7D; data not shown). These data strongly suggest that expression of the *M. truncatula SGL1* gene driven by the *SGL1* promoter, which is active in the initiating

leaf primordia and developing leaves, was not sufficient to alter the simple leaf structure in Arabidopsis.

## DISCUSSION

### The Role of *FLO/LFY* Orthologs in Compound Leaf Development

In most compound-leafed species, activation of *KNOX1* gene expression in initiating leaf primordia is correlated with development of compound leaves. In simple-leafed species, however, expression of the *KNOX1* genes is permanently down-regulated in the initiating leaf primordia in a process that requires MYB domain transcription repressors RS2 and AS1 in maize and Arabidopsis, respectively, and involves

hormonal signaling (Smith et al., 1992; Lincoln et al., 1994; Nishimura et al., 1999; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000; Ori et al., 2000; Sakamoto et al., 2001; Hay et al., 2002; Hay et al., 2006; Uchida et al., 2007; Guo et al., 2008). Molecular genetic studies suggest that regulatory processes leading to compound leaf development are more complex than that required for simple leaf development and involve multiple regulators such as the *KNOX1* homeobox genes, the TCP class transcription factor Lanceolate, whose activity is in turn regulated by miR319 and perhaps other yet-to-be-identified regulators (Bharathan and Sinha, 2001; Bharathan et al., 2002; Brand et al., 2007; Jasinski et al., 2007; Ori et al., 2007).

In compound-leafed legume species such as pea and alfalfa, in which the *KNOX1* genes are excluded from the leaf primordia and thus are not likely correlated with compound leaf development in these plants, the *FLO/LFY* orthologs appear to function in place of the *KNOX1* genes in control of compound leaf development (Champagne et al., 2007). Additional support for the role of *FLO/LFY* in compound leaf development comes from studies of tomato *fa* mutants and *L. japonicus pfm* mutants, in which the corresponding *FLO/LFY* orthologs are mutated (Molinero-Rosales et al., 1999; Dong et al., 2005), and from studies of downregulation of two *LFY* genes in soybean (Champagne et al., 2007). In tomato *fa* mutants, the number of small intercalary leaflets is reduced, although the number of primary leaflets is not affected (Molinero-Rosales et al., 1999). In *L. japonicus pfm* mutants, 99% of compound leaves are lacking one or two basal leaflets (Dong et al., 2005). In transgenic soybean lines in which the endogenous *LFY* genes are down-regulated, the leaflet number is slightly reduced (Champagne et al., 2007). Although these results collectively support a role for *FLO/LFY* in compound leaf development in tomato (Solanaceae species) and in *L. japonicus* and soybean (two legume species), the weaker leaf phenotypes of the *fa* and *pfm* mutants suggest that *FA* and *PFM* may play a minor role in compound leaf development. Because the weak leaf phenotype in *LFY* RNA interference transgenic soybean lines may be attributed to partial down-regulation of the endogenous genes, whether the two soybean *FLO/LFY* orthologs play a significant role in compound leaf development in soybean is not clear so far (Champagne et al., 2007).

By contrast, *M. truncatula sgl1* mutants exhibit strong compound leaf defects with all adult leaves changed to simple ones (Fig. 2), indicating that the *M. truncatula FLO/LFY* ortholog *SGL1* plays a significant role in the initiation of lateral leaflet primordia required for compound leaf development (Figs. 1 and 2). Similar to *sgl1* mutants, pea *uni* mutants also exhibit strong compound leaf defects, i.e. the number of leaflets is greatly reduced to one to three, and the terminal tendrils are completely missing. Genetic analyses of *uni*, *afila* (*af*), and *tendril-less* (*tl*) pea mutants lead to a hypothesis that *UNI* functions as a determinant of a central-fated shoot-like structure (Hofer and Ellis, 1998).

It is well established that *FLO/LFY* plays a key role in vegetative to reproductive transition, leading to flower development (Blazquez et al., 1997). This function is quite conserved across angiosperm species. Mutants of *FLO/LFY* orthologous genes are unable to complete the developmental transition, thus forming inflorescence-like structures in place of flowers (Coen et al., 1990; Weigel et al., 1992; Weigel and Nilsson, 1995; Hofer et al., 1997; Souer et al., 1998; Molinero-Rosales et al., 1999; Dong et al., 2005). The involvement of *FLO/LFY* in transition from development of indeterminate to determinate structures during the reproductive phase of growth suggests that *FLO/LFY* plays a role in promoting determinacy in inflorescence. The role of *SGL1* in the reproductive phase of growth appears to be contradictory with its role in compound leaf development in *M. truncatula*, because our data suggest that it promotes a transient phase of indeterminate growth (initiation of leaflet primordia). Future experiments are required to reconcile the discrepancy in the function of *SGL1* in floral and leaf development in *M. truncatula*. Interestingly, our hypothesis seems to be consistent with an earlier model, which postulates that *UNI* functions as a determinant for an indeterminate structure (central-fated shoot-like structure) during compound leaf development in garden pea (Hofer and Ellis, 1998). Furthermore, our hypothesis is consistent with the expression of *FLO/LFY* orthologs in leaf marginal blastozones (marginal areas with organogenetic activities) in a number of compound-leafed species, from which the leaflet primordia initiate (Hofer et al., 1997; Molinero-Rosales et al., 1999; Busch and Gleissberg, 2003). The transient nature of *SGL1* expression in developing leaves and the shortened petiole phenotype observed in *sgl1*, *uni*, and *pfm* mutants, as well as in transgenic soybean lines in which expression of two endogenous *LFY* genes are compromised, further support the role for *FLO/LFY* in promoting a transient phase of indeterminate growth, which is required for development of determinate structures, i.e. leaflets (Hofer et al., 1997; Dong et al., 2005; Champagne et al., 2007).

## Regulatory Aspects of Compound Leaf and Floral Development

The fact that proper expression of the Arabidopsis *LFY* rescued compound leaf and floral defects of the *sgl1* mutants and proper expression of the *M. truncatula SGL1* rescued the floral defects but did not alter the simple leaf structure of the *lfy* mutants unambiguously demonstrates that LFY and SGL1 are functional orthologs and that differences in leaf forms between the simple-leafed Arabidopsis and compound-leafed *M. truncatula* are not due to functional differences between LFY and SGL1. Rather, our results support a hypothesis that regulatory processes mediated by the SGL1 function are involved in initiation of leaflet primordia and development of compound leaves in *M. truncatula*.

Alterations in cis-regulatory sequences, e.g. 5′-upstream sequences, are also implicated in morphological variations during plant evolution (Busch and Gleissberg, 2003; Yoon and Baum, 2004; Baum et al., 2005; Maizel et al., 2005). Our data indicate that *SGL1* differs in its promoter activity from *LFY*, because only *SGL1* is highly expressed in developing leaves (Fig. 7). Additionally, *SGL1* is expressed in the entire SAM, including the central region, in contrast to *LFY*, whose expression is restricted to the periphery of SAM and excluded from the center of SAM (Weigel et al., 1992). A large variation in *LFY* expression pattern in the vegetative SAM has been reported across vascular plants (Busch and Gleissberg, 2003; Yoon and Baum, 2004; Baum et al., 2005). Currently, it is not clear whether variations in the expression of *LFY* orthologs in the vegetative SAM contribute to morphological variations during plant evolution. However, it was reported that prolonged expression of *LFY* in developing leaves appears to be associated with species with compound leaves (Busch and Gleissberg, 2003), consistent with the expression pattern of *SGL1* during compound leaf development in *M. truncatula* (Fig. 5).

In contrast to its role in compound leaf development being prominent only in legume species, particularly in the IRLC studied to date, the role of *FLO/LFY* in inflorescence and FM identity control has been shown to be highly conserved in flowering plants. In loss-of-function mutants of *FLO/LFY* orthologs, flowers exhibit homeotic defects, including lack of petals and stamens, and FMs being converted into inflorescence-like structures (Coen et al., 1990; Weigel et al., 1992; Hofer et al., 1997; Molinero-Rosales et al., 1999). The development of bilateral symmetric (zygomorphic) flowers in legume species, including *M. truncatula*, is quite different from development of Arabidopsis flowers with ancestral radial symmetry. In *M. truncatula* and many other species in the Papilionoideae subfamily of legumes, floral organs are initiated in the following order: sepals, carpel, petals, outer stamens, and inner stamens. Within each whorl, organs are initiated unidirectionally from the abaxial to adaxial direction. Furthermore, heterogeneous organs are initiated in the same whorl, and homogeneous organs are initiated in different whorls (Benlloch et al., 2003; Dong et al., 2005; Feng et al., 2006). By contrast, Arabidopsis and many other eudicot species initiate floral organs in a centripetal pattern in the following direction: sepals, petals, stamens, and carpel (Coen et al., 1990; Smyth et al., 1990; Bowman and Meyerowitz, 1991; Krizek and Fletcher, 2005). Despite dramatic differences in floral organ development among these diverse species, loss-of-function mutants of *FLO/LFY* orthologs exhibit similar homeotic defects. The observations that proper expression of *LFY* orthologous genes from flowering plants driven by the native *LFY* promoter functionally rescued *lfy* flower defects and expression of *LFY* rescued *sgl1* floral defects strongly support that *FLO/LFY* orthologs function in FM identity control during reproductive phase of growth (Coen et al., 1990; Weigel

et al., 1992; Molinero-Rosales et al., 1999; Dong et al., 2005). Future work intended to dissect regulatory processes mediated by the function of *FLO/LFY* orthologs promise to provide new insights on compound leaf development in legumes and molecular mechanisms that underlie morphological variations of vascular plants in general.

## MATERIALS AND METHODS

### Plant Materials and Growth Condition

*Medicago truncatula* ecotypes R108 and 'Jemalong' A17 were used in this study. NF740 (*sgl1-1*), NF1240 (*sgl1-2*), NF2138 (*sgl1-3*), and NF2703 (*sgl1-4*) alleles were isolated from a tobacco (*Nicotiana tabacum*) *Tnt1* retrotransposon tagged mutant collection of *M. truncatula* (d'Erfurth et al., 2003; Tadege et al., 2005, 2008). Initially, *M. truncatula* genotype R108 was transformed with a construct containing the complete *Tnt1* retroelement of tobacco as described (d'Erfurth et al., 2003). *Tnt1* tagging was carried out using one of these transgenic lines as starting material and tissue culture to activate *Tnt1* transposition. Mutagenized plants that contain multiple independent *Tnt1* inserts were regenerated from leaf explants of the starter line via somatic embryogenesis as described (Tadege et al., 2005, 2008). The four *sgl1* alleles were identified from screening of a segregating population of approximately 3,000 independent R1 lines. Arabidopsis (*Arabidopsis thaliana*) *lfy* heterozygous lines were isolated from a bulked population of SALK_057202 T-DNA insertion line. Plants were grown in MetroMix 350 soil mix in a greenhouse or a growth chamber with the following conditions: 24°C day/20°C night temperature; 16-h day/8-h night photoperiod; 70% to 80% relative humidity, and 150 $\mu$mol/m$^2$/s and 80 $\mu$mol/m$^2$/s light intensity for *M. truncatula* and Arabidopsis, respectively.

### SEM

Shoot apical and FMs were collected from shoot apices of wild-type and mutant plants 2 to 4 weeks and 2 to 3 months postgermination, respectively. Plant tissues were fixed by vacuum infiltration with 3.0% glutaraldehyde in 25 mM phosphate buffer, pH 7.0, for 1 h and then incubated in 4°C overnight. Plant tissues were further fixed with 1.0% osmium tetroxide in the same phosphate buffer overnight and then dehydrated in a graded ethanol series. Before electron scanning microscopic observations, tissues were critical-point dried in liquid $CO_2$, mounted on aluminum stubs, dissected, and sputter-coated with gold. Specimens were then examined under a Zeiss DSM-960A SEM (Carl Zeiss) at an accelerating voltage of 5 kV. Digital photographs were collected and assembled using Photoshop.

### Molecular Cloning of Full-Length *SGL1* and *MtSTP* Genes

To clone full-length *M. truncatula SGL1* (*MtUNI*) and *MtSTP* genes, we first searched all available *M. truncatula* sequences in GenBank against *UNI* and *STP*. The genomic sequence was available for *SGL1* in the database. Therefore, sequence alignments of the *SGL1* genomic sequence with *UNI/LFY/FLO* coding sequences allowed us to define the open reading frame of *SGL1*. In the case of *MtSTP*, no sequences were available in the database. Therefore, oligonucleotide primers were designed based on consensus sequences derived from *UFO*, *FIM*, and *STP* sequences. Total RNA was prepared from shoot apices of 2-week-old wild-type plants ('Jemalong' A17). RT-PCR was carried out by following the manufacturer's instructions (Invitrogen) using the following oligonucleotide primers: *SGL1*-forward: 5′-GCTTACCATGGA-TCCCGACGCATT-3′; *SGL1*-reverse: 5′-TAACTTAAAAAGGAAGGTGAG-CAGTTC-3′; *MtSTP*-forward: 5′-ATGGAAGGTTTTCACCCATCTATGA-3′; and *MtSTP*-reverse: 5′-CATAAGCAAAACCATGCAACTCAAAC-3′. PCR products were cloned into pGEM-T Easy vector (Promega) and sequences were confirmed by sequencing and deposited into the GenBank (AY928184 and AY932821).

We also PCR amplified and sequenced the *SGL1* genomic sequence from *M. truncatula* R108 ecotype. The corresponding sequence was also deposited into the GenBank (DQ672589).

## Identification of *Tnt1* Insertion Sites in *SGL1*

Genomic DNA samples were prepared from wild-type *M. truncatula* (R108), and *sgl1-1*, *sgl1-2*, *sgl1-3*, and *sgl1-4* alleles using a standard protocol. PCR amplification was carried out using either *SGL1* or *MtSTP* primers listed above. PCR amplification gave rise to a product 5.3 kb larger from *sgl1-1* to *-4* alleles than from the wild type when *SGL1* primers were used. We PCR amplified the *Tnt1* flanking sequences from *sgl1-1* to *sgl1-4* alleles using a combination of *SGL1* primers (see above) and *Tnt1* primers (*Tnt1*-upstream, 5′-CTCCAGACATTTTTATTTTTCACCAAG-3′; *Tnt1*-downstream, 5′-GCA-TTCAAACTAGAAGACAGTGCTACC-3′). These PCR products were fully sequenced.

## Generation of Plant Transformation Constructs

To generate the *SGL1* genomic clone for functional complementation of *M. truncatula sgl1* mutants, we first PCR amplified *SGL1* promoter sequence from wild-type *M. truncatula* ('Jemalong' A17) with the following primers: (*SGL1* promoter-forward) 5′-AATTGAATTCAAAAATGGTGTACCAAACATGAG-GTAGAA-3′ (an *Eco*RI site was introduced as underlined) and (*SGL1* promoter-reverse) 5′-CGTCGGGATCCATGGTAAGCAATG-3′ (a unique *Bam*HI site was highlighted). The PCR product was digested by *Eco*RI and *Bam*HI and cloned into the *Eco*RI-*Bam*HI sites of pCAMBIA3300 binary vector (the resulting construct was labeled as pCAMBIA3300-p*SGL1*). Similarly, we PCR amplified the *SGL1* coding sequence and 3′ untranslated region from the wild type with the following primers: (*SGL1*-forward) 5′-AGTTTCA-TTGCTTACCAT**GGATCC**-3′ (a unique *Bam*HI site was highlighted) and (*SGL1*-reverse) 5′-AATTCTGCAGAAAAATTAGCCTTTCCCATGCTAAAC-TTC-3′ (a *Pst*I site was introduced as underlined). The PCR product was digested by *Bam*HI and *Pst*I and cloned into the *Bam*HI-*Pst*I sites of the pCAMBIA3300-p*SGL1* construct. The resulting construct pCAMBIA3300-*SGL1* was sequenced and confirmed to contain no errors and was then introduced into disarmed *Agrobacterium tumefaciens* EHA105 and AGL1 strains via electroporation.

To generate the Arabidopsis *LFY* clone for complementation of *M. truncatula sgl1* mutants, we first recloned the *SGL1* promoter into the *Eco*RI-*Bam*HI sites of pCAMBIA3301. We then PCR amplified the full-length *LFY* cDNA from pDW123 (Blazquez et al., 1997) using the following primers: (*LFY*-forward) 5′-TTAT**GGATCC**TGAAGGTTTCACGAG-3′ (a unique *Bam*HI site was highlighted) and (*LFY*-reverse) 5′-AATTGGTCACCCTAGAAACGCAA-GTCGTCGC-3′ (the underlined sequence was an introduced *Bst*EII site). The PCR product was digested by *Bam*HI and *Bst*EII and subcloned into the *Bam*HI-*Bst*EII sites of the pCAMBIA3301-p*SGL1* construct. The resulting construct pCAMBIA3301-p*SGL1*::*LFY* was introduced into *A. tumefaciens* EHA105 and AGL1 strains via electroporation.

We generated pCAMBIA3301-p*SGL1*::*GUS* construct by restriction enzyme digestion of the pCAMBIA3301-p*SGL1* with *Nco*I and followed by religation, which removed cauliflower mosaic virus *35S* promoter between *SGL1* promoter and *GUS* gene from the vector. The resulting construct CAMBIA3301-p*SGL1*::*GUS* was introduced into *A. tumefaciens* EHA105 and AGL1 strains.

To clone the full-length *SGL1* cDNA for genetic complementation of Arabidopsis *lfy* mutants, we amplified the Arabidopsis *LFY* promoter from Columbia-0 wild-type plants by PCR using the following primers: (*LFY* promoter-forward) 5′-TATAGAATTCGTAATGGGCTGACCGAGAAGATAT-AAA-3′ (an *Eco*RI site was introduced as underlined) and (*LFY* promoter-reverse) 5′-CGTCGAAACCTTCAGGATCCCATAATCTA-3′ (a unique *Bam*HI site was highlighted). The PCR product was digested with *Eco*RI and *Bam*HI and ligated into the *Eco*RI-*Bam*HI sites of pCAMBIA3301, resulting in pCAMBIA3301-p*LFY*. The full-length *SGL1* cDNA was reamplified using the following primers: (*SGL1*-forward) 5′-AGTTTCATTGCTTACCAT**GGATCC**-3′ (a unique *Bam*HI site was highlighted) and (*SGL1*-reverse2) 5′-AATTGGT-CACCTAACTTAAAAAGGAAGGTGAGCAGTTC-3′ (a *Bst*EII site was introduced as underlined). The PCR product was digested by *Bam*HI and *Bst*EII and ligated into the *Bam*HI-*Bst*EII sites of pCAMBIA3301-p*LFY*. The resulting construct, pCAMBIA3301-p*LFY:SGL1*, was confirmed by sequencing and introduced into *A. tumefaciens* GV3101 strain.

## Stable Plant Transformation

*M. truncatula sgl1-1* (R108 ecotype) and R108 wild type were transformed with *A. tumefaciens* EHA105 or AGL1 strain harboring the various binary constructs described, using a transformation-regeneration protocol previously described (Chabaud et al., 1996; Trinh et al., 1998; Cosson et al., 2006). Transgenic T1 plants were genotyped using PCR with the following primers for amplification of the selection marker gene: *BAR*-forward, 5′-CCGTACCG-AGCCGCAGGAAC-3′ and *BAR*-reverse, 5′-CAGATCTCGGTGACGGGC-AGGAC-3′.

For Arabidopsis transformation, heterozygous (*lfy* +/−) plants (SALK_057202) were transformed by *A. tumefaciens* GV3101 strain harboring various binary constructs described, using the floral dipping method as previously described (Clough and Bent, 1998; Bent, 2006). T1 plants were selected on one-half strength Murashige and Skoog minimal organic medium supplemented with 1% agar and 5 μg/mL glufosinolate (PPT).

## RT-PCR

Total RNA was prepared using Tri Reagent (Molecular Research Center). Genomic DNA was removed using a DNA-free kit (Ambion). cDNA synthesis was performed using SuperScript II reverse transcriptase (Invitrogen) starting with 2 μg of total RNA in a 20-μL reaction with oligo(dT)$_{15}$ primers (Promega) at 42°C for 1 h. Primers 5′-AGACGCCTTGATGAAGAGGAAATTAA-3′ and 5′-TAGCAATTGCTTGAACCTGAATCAAG-3′ were designed to amplify 424 bp of the internal coding region of *SGL1*. Primers 5′-TCTTACTCTCAAG-TACCCCATTGAGC-3′ and 5′-GTGGGAGTGCATAACCTTCATAGATT-3′ were designed to amplify 329 bp *M. truncatula Actin* as an internal loading control. Primers 5′-CAGTGTCTGGATCGGAGGAT-3′ and 5′-TGAACAATC-GATGGACCTGA-3′ were designed to amplify the Arabidopsis *Actin* gene (At5G09810) as an internal loading control. The PCR was performed as follows: 2 min at 95°C, 25 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, and 5 min at 72°C. Amplified products were separated by electrophoresis according to their molecular weights.

## Phylogenetic Analysis

Phylogenetic analysis was performed using PAUP version 4 software (paup.csit.fsu.edu/). A maximum parsimony criterion was used to generate the single most parsimonious tree, with bootstrap values from 1,000 replicates and greater than 50% shown above the relevant branches.

## RNA in Situ Hybridization

RNA in situ hybridization was essentially carried out as previously described (Coen et al., 1990) with minor modifications. Briefly, *SGL1* transcripts were generated from a cDNA clone corresponding to the nucleotide 179 to 1,189 of the coding sequence and labeled with digoxigenin. Then 10-μm sections from shoot apices of 2-week-old R108 and *sgl1* mutants were processed and hybridized with digoxigenin-labeled sense and antisense probes followed by detection under the same condition.

## Histochemical GUS Staining

GUS staining was carried out as previously described (Shin et al., 2005) with modifications. Briefly, tissue samples were incubated in a GUS staining buffer (100 mM sodium phosphate, pH 7.0, 1 mM EDTA, 0.05% Triton X-100, 1 mM potassium ferricyanide and potassium ferrocyanide, and 1 mg/mL X-glucuronide) at 37°C overnight. Samples were then cleared in 50%, 70%, and 100% ethanol, rehydrated with 70% and 50% ethanol, and transferred to glycerol:ethanol solution (1:1, v/v) before microscopic examination. Images of GUS staining patterns were collected from a digital camera mounted on a dissection microscope (SMZ1500, Nikon) and assembled using Adobe Photoshop Elements 4.0 package.

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers AY928184, AY932821, and DQ672589.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Amino acid sequence alignments of SGL1 and its orthologs.

**Supplemental Table S1.** Genetic segregation analysis of the *sgl1-1* mutant suggests that *sgl1-1* segregates as a single recessive locus.

**Supplemental Table S2.** Distribution of the floral organ number in homozygous *lfy* transgenic plants compared with wild-type Columbia-0 plants.

## ACKNOWLEDGMENTS

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