LEUNIG, a putative transcriptional corepressor that regulates *AGAMOUS* expression during flower development

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Regulation of homeotic gene expression is critical for proper developmental patterns in both animals and plants. *LEUNIG* is a key regulator of the *Arabidopsis* floral homeotic gene *AGAMOUS*. Mutations in *LEUNIG* cause ectopic *AGAMOUS* mRNA expression in the outer two whorls of a flower, leading to homeotic transformations of floral organ identity as well as loss of floral organs. We isolated the *LEUNIG* gene by using a map-based approach and showed that *LEUNIG* encodes a glutamine-rich protein with seven WD repeats and is similar in motif structure to a class of functionally related transcriptional corepressors including Tup1 from yeast and Groucho from *Drosophila*. The nuclear localization of *LEUNIG–GFP* is consistent with a role of *LEUNIG* as a transcriptional regulator. The detection of *LEUNIG* mRNA in all floral whorls at the time of their inception suggests that the restricted activity of *LEUNIG* in the outer two floral whorls must depend on interactions with other spatially restricted factors or on posttranslational regulation. Our finding suggests that both animals and plants use similar repressor proteins to regulate critical developmental processes.

Arabidopsis flowers, like those of other dicots, are composed of four types of organs (sepals, petals, stamens, and carpels) arranged in four concentric whorls. Three classes of floral homeotic genes work in a combinatorial fashion to specify the identity of floral organs (1). These floral homeotic genes all encode DNA-binding transcriptional factors, which activate floral organ-specific developmental programs. Proper temporal and spatial expression of the C-class floral homeotic gene *AGAMOUS (AG)* is central to flower pattern formation. In wild-type, *AG* mRNA is expressed only in the inner two whorls of a flower to specify stamen and carpel development (2–4). In *leunig (lug)* and *apetala 2* (*ap2*) mutants, however, *AG* mRNA is ectopically expressed in the outer two whorls of a flower, resulting in the homeotic transformation from sepals to carpels and petals to stamens or absent (4, 5). Thus, the activities of *LUG* and *AP2* are required in the outer floral whorls to negatively regulate *AG* expression. In addition, *AG* RNA is expressed much earlier in *lug* and *ap2* mutant floral meristems, suggesting that *LUG* and *AP2* also regulate the temporal expression pattern of *AG*. A third gene, *CURLY LEAF*, is required to represses *AG* transcription mainly in vegetative tissues (6). Hence, transcriptional repression of *AG* is vital to both vegetative and reproductive development.

LUG provides a unique opportunity to study transcriptional repression in higher plants. Although *LUG* is required in the outer two whorls of a flower to repress *AG* mRNA expression, *LUG* is not required to specify sepal/petal identity in the outer two whorls because *ag lug* double mutants still develop normal sepals and petals (5). In contrast, *AP2*, which encodes two copies of a DNA-binding motif (7), is required to both repress *AG* and specify sepal/petal identity (3). Hence, unlike *AP2*, *LUG* is strictly a negative regulator of floral homeotic gene expression with no direct role in the specification of floral organ identity. In addition, *LUG* is distinct from *CURLY LEAF*, an *Arabidopsis* homolog of the *Drosophila* polycomb-group gene *Enhancer of zeste*, in that *LUG* is required for the initial repression of *AG* transcription at early stages of floral meristem development, whereas *CURLY LEAF* is required to maintain *AG* repression at later stages of flower development (6). Finally, *lug* mutants exhibit other defects that are independent of *AG*, including a split stigma (Fig. 1 *A* and *B*), abnormal carpel and ovule development, reduced female and male fertility, and narrower leaves and floral organs (5, 8–10). Therefore, *LUG* likely regulates the expression of several target genes in diverse developmental processes.

To further understand the molecular mechanisms on how *LUG* represses *AG* expression, we isolated the *LUG* gene by using a map-based approach. Unlike other genes known to regulate floral homeotic gene expression, *LUG* does not encode any obvious DNA-binding motifs. Instead, *LUG* encodes a glutamine-rich protein with seven WD repeats at the COOH terminus. *LUG* is similar to the yeast Tup1 and *Drosophila* Groucho (Gro) transcriptional corepressors both in genetic function and in motif structure. Because transcriptional repression clearly plays a major role in higher plant development, our study may contribute to the understanding of general regulatory mechanisms of higher plant development.

Methods

Mutant Strains and Mapping Population. *lug-1* and *lug-3* have been described (5). *lug-12* and *lug-16* are ethyl methanesulfonate (EMS)-induced mutation in the Landsberg *erecta* (L-*er*) background. *lug-14* is a Ds transposon-induced allele in L-*er*. Two mapping populations were screened to identify recombinant chromosomes in F_2 or F_3 by using the genetic markers *cer2* and *ap2* that flank *LUG* at 5 and 16 map units, respectively. A *cer2* allele in Wassilewskija ecotype was crossed into *lug-1* in L-*er* ecotype. F_2 *cer2* families and F_2 *lug-1* families were screened for segregating *cer2 lug-1* double mutants in F_3. These double mutants were used for subsequent fine mapping. A second mapping population was similarly constructed by crossing an *ap2* mutant in Columbia ecotype with *lug-8* in L-*er* ecotype.

Positional Cloning of *LUG*. Fifteen recombinants between *cer2* and *lug* and 52 recombinants between *ap2* and *lug* were further

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Abbreviations: *AG*, *AGAMOUS*; *lug*, *leunig*; *ap2*, *apetala 2*; EMS, ethyl methanesulfonate; L-*er*, Landsberg *erecta*; GFP, green fluorescent protein; Gro, Groucho.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF277458).

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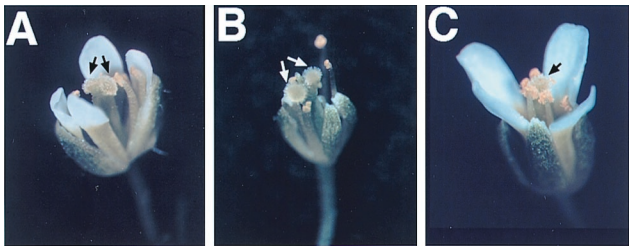


Fig. 1. Micrographs of *lug* flowers. (*A*) *lug-16*, a weak allele. The pair of arrows indicate the split stigma characteristic of *lug* mutant flowers. (*B*) *lug-12*, a strong allele. The flower has no petal and exhibits split stigma (arrows). The smaller size sepals are caused by partial homeotic transformation into carpelloid sepals. (*C*) The flower of a *lug-16* mutant rescued by cosmid 31-G. The flower and its stigma are similar to wild type.

screened with cleaved amplified polymorphic sequence (CAPS) markers shown in Fig. 2*A*. The CAPS markers F10N7H and F4D11S flank *lug* with one recombinant on each side. Information for these markers is at http://www.arabidopsis.org/maps/CAPS_Chr4.html. The region from F10N7H to F4D11S is covered by the yeast artificial chromosome (YAC) clone CIC1F9 and five overlapping bacterial artificial chromosome (BAC) clones (Fig. 2*A*). A cosmid library was made from the YAC CIC1F9 and the binary cosmid transformation vector pCLD04541. The procedure is essentially the same as described (11). Cosmid clones were isolated by screening the cosmid library with each of the five BAC clones as probes. The cosmid contig, which spans 284 kb, was constructed by using restriction enzyme analyses. A total of 23 cosmids were transformed into the weak and fertile *lug-16* mutants via *Agrobacterium* strain GV3101 and vacuum infiltration (12). Complementation rescue by the 31–1 and 31-G cosmid clones was observed by the wild-type primary transgenic plants and subsequently was verified in the next generation by 100% ($n = 44$) cosegregation of the rescued plants with corresponding cosmid clones.

Isolation and Analyses of *LUG* mRNA. Three *LUG* cDNA clones were isolated by screening a total of ≈500,000 plaques from two cDNA libraries: Weigel L-*er* flower library and Kieber and Ecker Columbia cDNA library (3–6 kb). A 5′ rapid amplification of cDNA ends kit version 2.0 (GIBCO/BRL) was used to identify the 5′ sequence of *LUG* mRNA. Three nested primers from the 5′ gene-specific region of *LUG* cDNA were used. They are 5′-TCTCATTGGTCCTAGC-3′, 5′-CCAGAAGACAGACCACCACTC-3′, and 5′-CAGGTGCGTCAATAGCAACTG-3′. The PCR products were cloned into pGEM-T vector (Promega) and sequenced.

For Northern analyses, poly(A)$^+$ RNA was isolated from respective tissues by using the FASTTRACK 2.0 RNA isolation system (Invitrogen). Both root and shoot mRNA was isolated from the same 10-day-old seedlings. mRNA from the stems, cauline leaves, and inflorescences was isolated from the same 21-day-old plants that had just bolted. mRNA from the *lug-1*, *lug-12*, and *lug-16* was isolated from inflorescences of 21-day-old plants. A total of ≈1 μg poly(A)$^+$ RNA was loaded per lane and fractionated in a 1.2% agarose gel. The Northern filters were probed with the 3′ 520-bp *LUG* cDNA sequence. The actin probe is a 1.6-kb actin cDNA from *Brassica oleracea* (13). The northern images were generated by using a Cyclone Storage Phosphor System (Packard) and quantitated by using OPTIQUANT IMAGE ANALYSIS software (Packard).

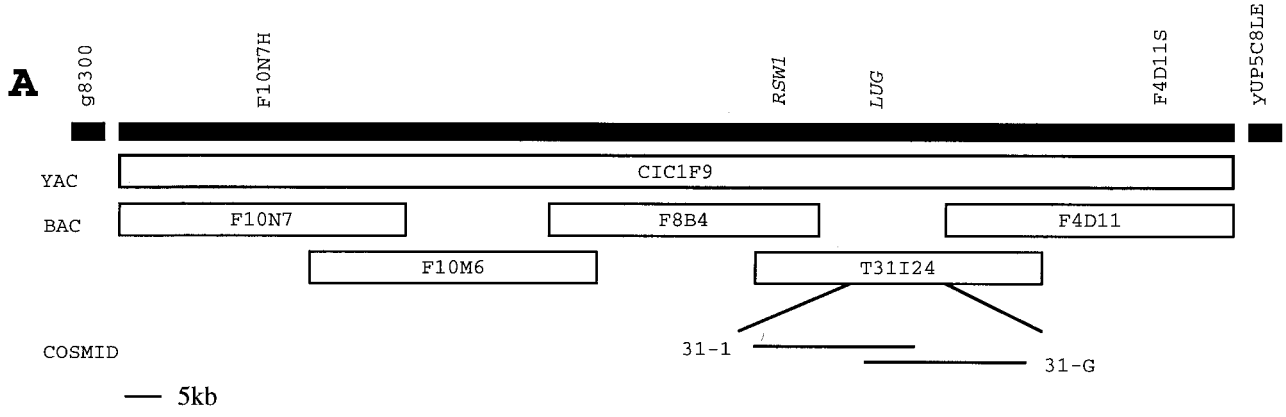
The *in situ* sense and antisense probes were derived from a 220-bp *Eco*RV/*Ssp*I fragment from the 5′ untranslated region of *LUG*, which was cloned into the pSK vector (Stratagene). The probes were labeled with digoxigenin-UTP as described (14), and the fixation and hybridization procedure was based on www.wisc.edu/genetics/CATG/barton/protocols.html.

Green Fluorescent Protein (GFP)–LUG Chimeric Gene Construction and Transient Expression. A full-length *LUG* cDNA was PCR-amplified with primers: 5′-GAGATCTATGTCTCAGACCAACTG-3′ and 5′-TAAGATCTTCGTCAGATCATACAACAAC-3′ by using the Platinum High Fidelity *Taq* polymerase (GIBCO/BRL). The amplified fragment was cloned into the pGEM-T vector (Promega) and sequenced. Full-length *LUG* cDNA in the pGEM-T vector was digested with *Bgl*II and cloned into the pAVA393 vector (15). The resulting plasmid construct, verified by sequence analyses, carries an in-frame fusion of *LUG* to the carboxyl terminus of GFP. Transient expression assays in onion epidermal cells were carried out by using a Biolistic Particle Delivery System (Bio-Rad) (16), and the tissues were visualized and photographed as described (14). Images were obtained with a ×20 objective and a Nikon 2000 35-mm camera.

Results

Positional Cloning of *LUG*. We isolated the *LUG* gene by positional cloning. The position of *LUG* on chromosome 4 was established by using recombinant chromosomes and PCR-based markers (Fig. 2*A* and *Methods*). A 284-kb cosmid contig of 23 cosmid clones was transformed into *lug-16* mutants to test for rescue of the mutant phenotype. *lug-16*, a weak *lug* mutant with the highest fertility among all *lug* alleles, allows the direct transformation of homozygous *lug-16* plants. Two overlapping cosmid clones, 31–1 and 31-G, complemented the *lug-16* mutant as shown by the wild-type flowers formed by both primary and secondary transgenic plants (Figs. 1 *A* and *C* and 2*A*; *Methods*). The overlapping region of these two cosmids contains one gene that spans the genomic sequences from nucleotides 23704 to 29200 of cosmid L23H3, whose sequence was released by the European Union *Arabidopsis* Sequence Consortium (AL050398).

Three cDNA clones residing in the overlapping region of the two cosmids were isolated by screening cDNA libraries (*Methods*). 5′ Rapid amplification of cDNA ends was used to identify the 5′ sequences of the mRNA (*Methods*). Sequence analyses of five *lug* mutant alleles all revealed single base changes in this gene. *lug-3* and *lug-12* mutants both exhibit strong phenotypes (ref. 5; Fig. 1*B*), and both possess a C to T change that results in a glutamine (Q) to a stop codon near the NH_2 terminus (Fig. 2*B*). Northern analyses of *lug-12* mRNA revealed that the level of *lug-12* transcript is only at about 38% of the wild-type level (Fig. 3*A*, lane 7), indicating that the premature termination of translation may have caused a reduced stability of the *lug-12* transcript. *lug-16* causes a G to A change at the splicing acceptor site of the third exon. This single base substitution apparently alters splicing as shown by a reduced size of *lug-16* transcript (Fig. 3*B*, lane 8). However, the level of the abnormally spliced *lug-16* transcript is increased to about 200% of wild-type level (Fig. 3*B*). One possible explanation may be that *LUG* is involved in repressing its own transcription and that an increased *LUG* mRNA level is seen only in those *lug* mutants that do not suffer from premature translational termination. The intermediate-strength *lug-1* mutation causes a G to A change at the splicing acceptor site of the eighth exon. Sequence analyses of reverse transcription–PCR products indicated that the *lug-1* transcript uses an alternative splice acceptor site only one base 3′ to the original site (data not shown). This results in a frame shift followed by 44 new amino acids and then a stop. Northern analyses revealed that the level of the *lug-1* transcript is reduced to 45% of wild-type level (Fig. 3*A*, lane 6), probably because of the premature translational termination. Finally, the strong *lug-14* mutation inserts an A and changes the last 39 amino acids of the protein (Fig. 2*B*). These data indicated that we had isolated the *LUG* gene.



B

* *lug-16* splicing

MSQTNWEADKMLDVYIHDYLVKRDLKATAQAFQAEGKVSSDPVAIDAPGGFLFEWWSVFWDIFIARTNEKHSEVAASYIETQMIKARE**QQLQQSQHPQV**

* *lug-12* stop * *lug-3* Stop

SQQQQQQQQQQIQMQQLLLQRAQQQQQQQQQQHHHHQQQQQQQQQQQQQQQQQQQQQHQNQPPSQQQQQQSTPQHQQQPTPQQQPQRRDGSHLANGSANG

LVGNNSEPVMRQNPGSGSSLASKAYEERVKMPTQRESLDEAAMKRFGDNVGQLLDPSHASILKSAAASGQPAGQVLHSTSGGMSPQVQTRNQQLPGSAV

* *lug-1* splicing

DIKSEINPVLTPRTAVPEGSLIGIPGSNQGSNNLTLKGWPLTGFDQLRSGLLQQQKPFMQSQSFHQLNMLTPQHQQQLMLAQQNLNSQSVSEENRRLKM

LLNNRSMSLGKDGLGSSVGDVLPNVGSSLQPGGSLLPRGDTDMLLKLKMALL**QQQQQHQQQGGGNPPQPQPQPQ**PLNQLALTNPQPQSSNHSIHQQEKL

GGGGSITMDGSISNSFRGNEQVLKNQSGRKRKQPVSSSGPANSSGTANTAGPSPSSAPSTPSTHTPGDVISMPNLPHSGGSSKSMMMFGTEGTGTLTSP

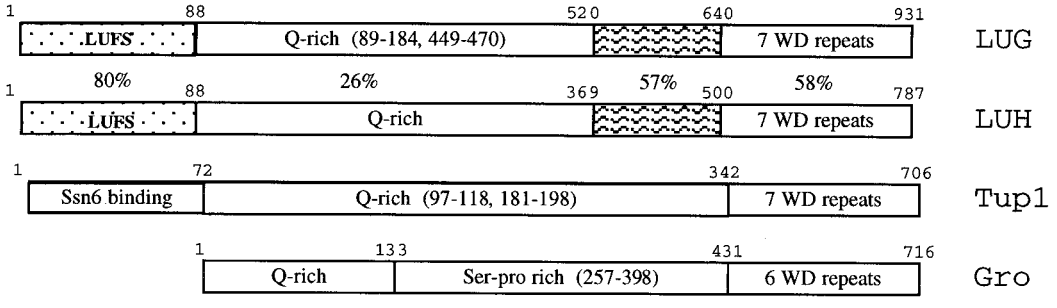
SNQLADMDRFVEDGSLDDNVESFLSQEDGDQRDAVTRCMDVSKGFTFTEVNSVRASTTKVTCCHFSSDGKMLASAGHDKKAVLWYTDTMKPKTTLEEHT

AMITDIRFSPSQLRLATSSFDKTVRVWDADNKGYSLRTFMGHSSMVTSLDFHPIKDDLICSCDNDNEIRYWSINNGSCTRVYKGGSTQIRFQPRVGKYL

AASSANLVNVLDVETQAIRHSLQGHANPINSVCWDPSGDFLASVSEDMVKVWTLGTGSEGECVHELSCNGNKFQSCVFHPAYPSLLVIGCYQSLELWNM

* *lug-14* frame shift

SENKTMTLPAHEGLITSLAVSTATGLVASASHDKLVKLWK



D

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LUG       12 LDVYIHDYLVKRDLKATAQAFQAEGKVSSDPV       AIDAPGGFLFEWWSVFWDIFIAR 66
LUH       12 LDVYIYDYLVKKKLHNTAKSFMTEGKVSSDPV       AIDAPGGFLFEWWSVFWDIFIAR 66
Flo8      77 LNEYIFDFLTKSSLKNTAAAFAQDAHLDRDKG (X)21 VVDTPQGFLYEWWQIFWDIFNTS 152
AF077048  22 LALYVYEYLLHVGAQKSAQTFLSEIRWEKNIT        LGEPPGFLHSWWCVFWDLYCAA 75
                *  *: : * :  :  :*  *                 : * *** :** :***::
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Fig. 2. Molecular cloning of *LUG*. (*A*) A physical map of *LUG* on chromosome 4. Open bars represent yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) clones. The relative position of several PCR-based markers is placed above the chromosome. Cosmids 31–1 and 31-G both complemented the *lug-16* mutant. (*B*) The protein sequence of *LUG* from L-*er*. The position of five *lug* mutations is indicated above the amino acid sequence. The two glutamine-rich regions are in bold. The seven WD repeats are underlined and are identified by using http://pfam.wustl.edu/hmmsearch.shtml. The specific base pair changes in each *lug* allele are described below based on the sequences of cosmid L23H3 (AL050398). *lug-3* causes a C to T change at 25309; *lug-12* causes a C to T change at 25198; *lug-16* causes a G to A change at 24693; *lug-1* mutation causes a G to A change at 26267; *lug-14* mutation inserts an A after 29080. (*C*) Structural similarity

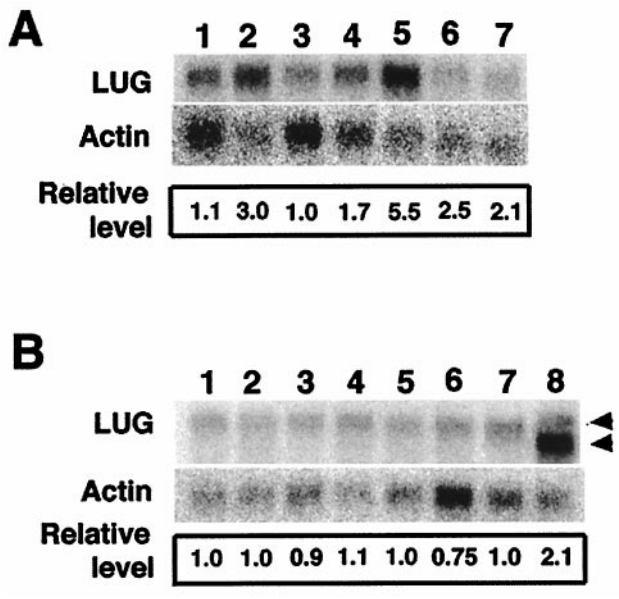

Fig. 3. LUG mRNA expression. (*A*) *LUG* mRNA is expressed in all tissues including roots (lane 1), shoots (lane 2), stems (lane 3), cauline leaves (lane 4), and inflorescences (lane 5). A decreased level of *LUG* mRNA is detected in *lug-1* (lane 6) and *lug-12* (lane 7) mutant inflorescences. The mRNA level is corrected with the actin loading control, and the relative level is derived by comparing all signals to the stem (lane 3), which equals to 1.0. (*B*) *LUG* mRNA expression in inflorescences of wild-type (lane 1), *ap1–1* (lane 2), *ap2–1* (lane 3), *ap2–2* (lane 4), *pi-1* (lane 5), *ag-2* (lane 6), *lfy-6* (lane 7), and *lug-16* (lane 8). With the exception of ag-2, *LUG* mRNA level in most mutants appears unchanged. In *lug-16*, *LUG* transcript is reduced in size and increased in abundance (arrowheads).

LUG Protein Is Q-Rich and Contains Seven WD Repeats. Based on the *LUG* cDNA and 5′ rapid amplification of cDNA ends, the *LUG* gene encodes a protein of 931 aa (Fig. 2*B*). Near the NH_2 terminus of LUG, two Q-rich regions are identified (Fig. 2*B*). The first Q-rich region (residues 89–184) contains 66 Qs, including three uninterrupted stretches of 10, 10, and 20. The second Q-rich region (residues 449–470) contains 12 Qs. The COOH terminus of the protein contains seven WD repeats (Fig. 2*B*). Finally, the region between the Q-rich and the WD repeats is rich in serine, glycine, leucine, and proline.

BLAST search revealed an *Arabidopsis* chromosome 2 gene with the highest sequence similarity to *LUG*. This homolog of *LUG* (named *LUH;* AC 003974) exhibits 44% overall sequence identity to *LUG* (Fig. 2*C*). The presence of several *Arabidopsis LUH* expressed sequence tag clones indicates that *LUH* is an expressed gene. The NH_2-terminal 88 aa of *LUG* show 80% identity to *LUH*. This high level of conservation suggests that the NH_2-terminal 88 aa may define an important functional domain. Database search with the NH_2-terminal 88 aa revealed 35% and 31% sequence identity to the yeast Flo8 gene (17, 18) and a human single-strand DNA-binding protein (AF077048), respectively (Fig. 2*D*). Thus, we named this conserved domain LUFS (LUG/LUH, Flo8, single-strand DNA-binding protein), which is also present in genes of unknown function in organisms such as *Schizosaccharomyces pombe*, *Drosophila*, and *Caenorhabditis elegans*. The functional nature of this domain is unknown.

The second motif of LUG that shows sequence similarities to other proteins in databases is the WD repeat. Among WD repeat-containing proteins, the yeast transcriptional corepressor Tup1 (19) exhibits additional levels of similarity to *LUG*. Tup1 protein shows 21% overall sequence identity to LUG. Like LUG, Tup1 protein has Q-rich regions near the NH_2 terminus and seven WD repeats at the COOH terminus (Fig. 2*C*). However, Tup1 has a unique NH_2-terminal domain of 72 aa, which bears no sequence similarity to the LUFS domain of *LUG.* Tup1 belongs a class of functionally related transcriptional corepressors including *Drosophila* Gro, which also possesses a NH_2-terminal Q-rich region and COOH-terminal WD repeats (Fig. 2*C*; refs. 20 and 21).

***LEUNIG* Is Expressed in Both Vegetative and Reproductive Tissues.** Because *lug* mutants exhibit defects in both reproductive and vegetative tissues, we examined *LUG* mRNA expression in different plant tissues and organs by Northern blots. We found that *LUG* is expressed in all tissues tested including roots, shoots, stems, cauline leaves, and inflorescences (Fig. 3*A*). The expression level is highest in inflorescences that contain flowers at all stages. The broad mRNA expression profile of *LUG* is consistent with a role of *LUG* in regulating multiple developmental processes. To identify transcriptional regulators of *LUG*, we examined *LUG* mRNA expression in several floral homeotic mutants including *apetala1* (*ap1*), *ap2, pistillata (pi),* and *ag* (2–3, 22) and in floral meristem identity mutant *leafy* (*lfy*) (23). Using mRNAs isolated from floral tissues, we found that, with the exception of *ag-2*, *LUG* mRNA level appeared unchanged in all of the mutants tested (Fig. 3*B*). In the *ag-2* mutants (Fig. 3*B*, lane 6), however, *LUG* mRNA level is slightly reduced. Because the *ag-2* mutant flowers do not form any stamens and carpels, which express high levels of *LUG* mRNA (see below), it is not surprising that *LUG* mRNA appeared reduced in the *ag-2* mutants.

In situ hybridization was used to examine further the *LUG* mRNA expression pattern in young 14-day-old seedlings and during flower development (Fig. 4). A low level of *LUG* mRNA was detected both in shoot apical meristems and inflorescence meristems (Fig. 4 *A* and *D*). *LUG* mRNA level is low in the first few young leaf primordia in seedlings but increases rapidly in older leaves (Fig. 4*A*). In cauline leaves of bolted plants, *LUG* mRNA is more abundant at the adaxial side of the leaves (Fig. 4*C*). In stems and carpel valves, *LUG* mRNA is prominently expressed in the vascular tissues (Fig. 4 *C*, *G*, and *I*). Because one of the most important function of *LUG* is to repress *AG* RNA expression in the outer two whorls of a flower, we tested whether *LUG* RNA is expressed only in the outer two floral whorls. We found that *LUG* mRNA is detected in all four floral whorls at the time of their inception (Fig. 4 *C–F*). *LUG* is highly expressed in the entire stage 1–2 floral primordia, and in young floral organ primordia (sepals, petals, stamens, and carpels) (Fig. 4 *C–F*). Thus, the ability of *LUG* to repress *AG* expression in the outer two whorls must depend on either posttranslational regulation or interaction with other spatially restricted regulators. During later stages of flower development, *LUG* expression subsides from sepals (at stage 7) and is detected in the developing stamen and carpel primordia (Fig. 4*F*). Interestingly, the expression of *LUG*

between *LUG*, *LUH* (AC 003974), the *S. cerevisiae* Tup1 (19), and the *Drosophila* Gro (20). Numbers above and in parentheses correspond to amino acids. The percentage between the amino acid numbers indicates the level of identity between *LUG* and *LUH*. The LUFS domain in LUG and LUH is indicated by a dotted region. A second highly conserved region between LUG and LUH immediately precedes the WD repeats and is marked by wavy lines. The level of similarity is identified by using http://www.bioinformatix.com/sas/. (*D*) Sequence alignment in the LUFS domain among LUG, LUH (AC003974), Flo8, and a human single-strand DNA-binding protein (AF077048). Numbers correspond to amino acids. Flo8 has extra 21 aa indicated by (X)21. Amino acids conserved in all four genes are in bold and are indicated by *. Amino acids conserved in three of the four genes are in bold and are indicated by :.

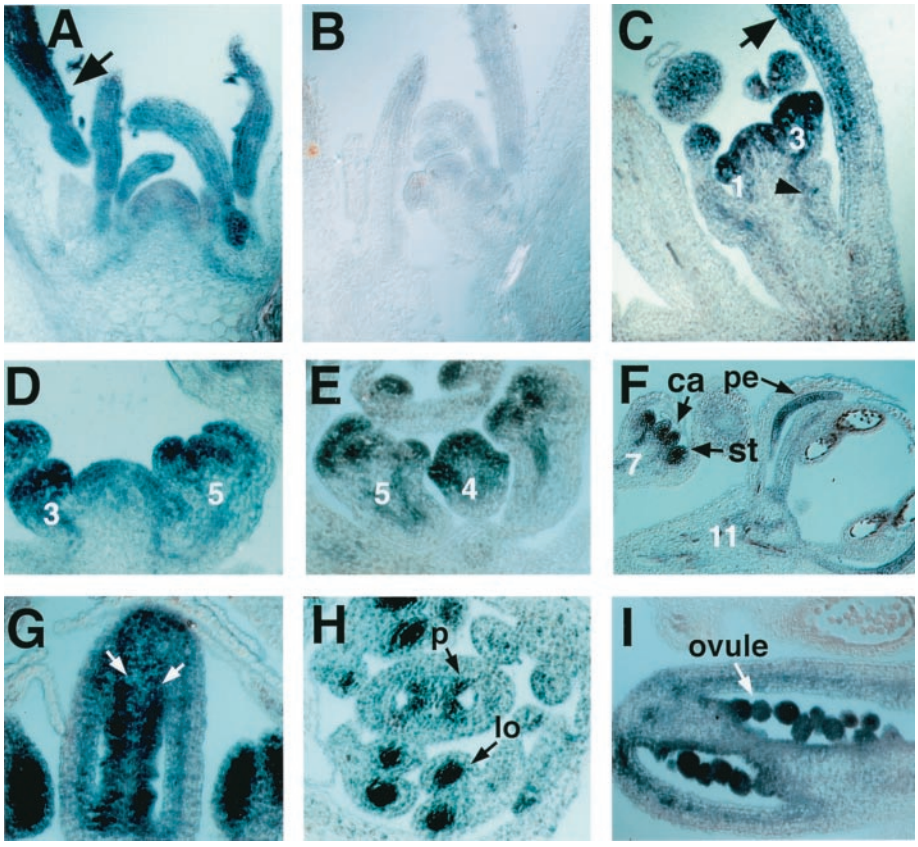

Fig. 4. *In situ* expression pattern of *LUG* mRNA. (*A*) *LUG* expression in 14-day-old seedlings. A low level of *LUG* mRNA is present in the shoot apical meristem and in the first few young leaves. *LUG* mRNA level increases dramatically in more developed leaves (arrow). (*B*) *LUG* sense probe to 14-day-old seedlings. (*C*) *LUG* mRNA is detected in the secondary inflorescence meristem and in the stage 1 and 3 floral meristems (numbers indicate the stage of respective floral meristems; ref. 33). *LUG* mRNA is detected in vascular tissues (arrowhead). *LUG* mRNA level is also more abundant in the adaxial side of the cauline leaf (arrow). (*D*) *LUG* mRNA level is low in the inflorescence meristem but increases in young flowers (stages 3 and 5, respectively). (*E*) *LUG* is strongly expressed in the sepal primordia and the central dome of the stage 4 and 5 flowers. (*F*) At stage 7, *LUG* mRNA is not detected in sepals, but is present in the carpel (ca) and stamen (st) primordia. *LUG* mRNA is persistently detected in petals (pe) as shown in this stage 11 flower. (*G*) In stage 10 carpels, *LUG* mRNA is strongly expressed in the placenta/ovule primordia (arrows) and weakly expressed in the carpel valves. (*H*) A cross section of a stage 9 flower. A high level of *LUG* mRNA is detected in placenta (p) and locules (lo) of the anther. (*I*) *LUG* mRNA is detected in developing ovules at stage 12.

in petals persists until at least stage 12 (Fig. 4*F*). In addition, *LUG* mRNA is found prominently in the placenta/ovules (Fig. 4 *G–I*) and in locules of anthers (Fig. 4*H*). This high level of *LUG* mRNA expression during both female and male gamete development suggests that *LUG* plays an important role in ovule and pollen development and is consistent with the observed abnormality in *lug* female and male fertility (5, 8–10).

GFP–LUG Fusion Protein Is Localized in the Nucleus. If *LUG* functions as a transcriptional regulator, LUG protein should be located in the nucleus. However, *LUG* does not appear to encode any obvious nuclear localization signal (Fig. 2*B*). To determine the subcellular location of LUG, we made a fusion protein between LUG and the GFP. A full-length *LUG* cDNA was inserted COOH-terminal to the GFP, and the fusion construct was transiently expressed in onion epidermal cells. The GFP–LUG fusion protein localizes to the nucleus of onion epidermal cells (Fig. 5 *A* and *C*), whereas GFP alone localizes to both cytoplasm and nucleus (Fig. 5 *B* and *D*). The nuclear localization of GFP-LUG supports *LUG* as a transcriptional regulator.

Discussion

Our molecular isolation of *LUG* reported here indicates that unlike other regulators of floral homeotic genes such as *AP2* and *ANT* (7, 24, 25), *LUG* does not possess any obvious DNA-binding motifs. Instead, *LUG* encodes seven WD repeats, a LUFS motif, and two Q-rich regions. The WD repeats, which mediate protein–protein interactions, are found in proteins with

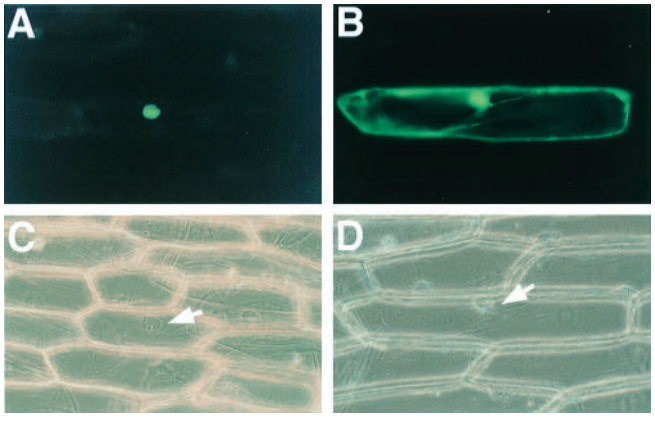

Fig. 5. Nuclear localization of GFP-LUG. (*A* and *B*) Dark-field images. (*C* and *D*) Corresponding phase-contrast microscopic images. (*A* and *C*) An onion epidermal cell transiently expressing the GFP-LUG chimeric protein. (*B* and *D*) An onion epidermal cell transiently expressing the GFP protein (vector pAVA 393; ref. 15). Arrows mark the location of nucleus.

a wide variety of biochemical functions (26). Our finding of the single base insertion that eliminates the last WD repeat in the strong *lug-14* mutant suggests that the last WD repeat is indispensable for the function of *LUG*. Although the function of LUFS motif is unknown, the *S. cerevisiae* Flo8, which also possesses the LUFS domain near its NH_2 terminus, is a transcriptional activator of Flo1 (17). However, there is no evidence that Flo8 binds to Flo1 DNA directly, and the LUFS domain is unlikely a DNA-binding motif. Hence, it is highly unlikely that *LUG* directly interacts with *AG* cis-regulatory elements to repress *AG* transcription, and LUG must interact with or recruit other proteins to exert its negative effect on transcription.

***LUG* Is Similar in Motif Structure to the Yeast Corepressor Tup1.** The overall motif structure of *LUG* is similar to a class of functionally related transcriptional corepressors, including the yeast Tup1, *Drosophila* Gro, and mammalian TLE (transducin-like enhancer of split) (Fig. 2*C*; refs. 19–21 and 27). The mechanism of how this class of transcriptional corepressors regulates target gene expression is relatively well understood (reviewed in refs. 21 and 27). Tup1 is brought to target promoters by sequence-specific DNA-binding proteins, and Tup1 regulates a wide array of independent sets of genes such as **a**-cell-specific genes, glucose-repressed genes, flocculation genes (such as Flo1), and DNA damage-induced genes (27, 28). The NH_2-terminal 72 aa of Tup1 forms repression complexes with Ssn6, a tetratricopeptide repeat protein (29). Interactions between the Tup1/Ssn6 complex and the specific DNA-binding factors (via the Tup1 WD repeats) bring about transcriptional repression to specific target genes. For example, the homeodomain protein α2 and the MADS box protein Mcm1 bind cooperatively to the promoters of **a**-cell-specific genes and recruit the Tup1/Ssn6 complex to repress **a**-cell-specific genes (30). Likewise, the *Drosophila* Gro, which possesses a Q-rich region at the NH_2 terminus and WD repeats at the COOH terminus (Fig. 2*C*), is a maternally contributed protein that interacts with a variety of regulatory proteins such as Hairy, Dorsal, and Engrailed to repress different target genes expression during segmentation, dorsal-ventral patterning, and neurogenesis (21).

A Proposed Model on How *LUG* Regulates AG. With the molecular isolation of *LUG*, we can begin to understand the mechanism of how *LUG* regulates the spatial and temporal expression pattern of *AG* and in what context *LUG* exerts its functional specificity in different tissues. The similarity in structure, transcriptional repression, and the regulation of multiple processes suggests that *LUG* may function via a similar mechanism as Tup1 and Gro. In outer whorls of *Arabidopsis* flowers, *LUG* may exert its transcriptional repression by interacting with transcription factors that bind to the cis-regulatory elements of *AG*. *AP2* and the more recently identified *Sterile Apetala* (*SAP*) (31), for example, could be such candidate transcription factors that mediate the repression by *LUG*. In particular, *ap2* and *lug* mutations exhibit synergistic interactions with respect to defects in *AG* repression (5), and both *AP2* and *LUG* were shown to repress *AG* through the same enhancer sequences that span most of the second intron of *AG* (32). However, *in vitro* coprecipitation assays failed to detect physical interactions between LUG and AP2 (unpublished work). Either *AP2* does not directly mediate the effect of *LUG* or additional proteins may be required for LUG to physically interact with AP2. Ubiquitously distributed *LUG* and *AP2* mRNA in all four floral whorls (Fig. 4; ref. 7) points to the need of other whorl-specific factors to confer their spatially restricted activity. In addition, because of the diverse functions of *LUG* revealed by its phenotype and by its broad mRNA expression profile, *LUG* may interact with several different DNA-binding factors in different tissues or at different developmental stages to confer its regulatory specificity. The molecular isolation and analyses of *LUG* provided us with a unique opportunity to further investigate mechanisms underlying spatially and temporally restricted gene expression and to identify interacting genes.

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