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

Joann Conner* and Zhongchi Liu*†

*Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742; and †Horticulture Department, University of Georgia, Coastal Plains Experimental Station, Tifton, GA 31793

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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 deletion 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 (sepalas, 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 stamens 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 repress 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 F2 or F3 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. F2 cer2 families and F2 lug-1 families were screened for segregating cer2 lug-1 double mutants in F3. 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. AF217458).

*To whom reprint requests should be addressed. E-mail: Z117@umail.umd.edu.

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A total of 23 cosmids were transformed into the pGEM-T vector (Promega) and sequenced. Full-length LUG cDNA in the pGEM-T vector was digested with BglII and cloned into the pAVA939 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. 2A and Methods). A 284-kb cosmide contig of 23 cosmids 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 cosmids 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. 1A and C and 2A; 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. 1B), and both possess a C to T change that results in a glutamine (Q) to a stop codon near the NH2 terminus (Fig. 2B). 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. 2B, 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. 3B, lane 8). However, the level of the abnormally spliced lug-16 transcript is increased to about 200% of wild-type level (Fig. 3B).

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 result 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. 3A, 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. 2B). These data indicated that we had isolated the LUG gene.

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 10-day-old seedlings. mRNA from the stems, cauline leaves, and inflorescences was isolated from the same 10-day-old seedlings. mRNA from the stems, cauline leaves, and inflorescences was isolated from the same 10-day-old seedlings. mRNA from the stems, cauline leaves, and inflorescences was isolated from the same 10-day-old seedlings. RNA was isolated from the 21-day-old plants that had just bolted. mRNA from the cauline leaves, and inflorescences was isolated from the same respective tissues by using the FASTTRACK 2.0 RNA isolation kit (GIBCO/BRL). Both root and shoot mRNA was isolated by using recombinant chromosome and PCR-based markers (Fig. 2A and Methods). A 284-kb cosmide contig of 23 cosmids 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 cosmids 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. 1A and C and 2A; 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).

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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. 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 25080. (C) Structural similarity
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 NH₂ terminus and seven WD repeats at the COOH terminus (Fig. 2C). However, Tup1 has a unique NH₂-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 NH₂-terminal Q-rich region and COOH-terminal WD repeats (Fig. 2C; 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. 3A). 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. 3B). In the ag-2 mutants (Fig. 3B, 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. 4A and D). LUG mRNA level is low in the first few young leaf primordia in seedlings but increases rapidly in older leaves (Fig. 4A). In cauline leaves of bolted plants, LUG mRNA is more abundant at the adaxial side of the leaves (Fig. 4C). In stems and carpel valves, LUG mRNA is prominently expressed in the vascular tissues (Fig. 4C, G, and I). Because one of the most important function of LUG is to repress AG mRNA 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. 4C–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 whors 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. 4F). Interestingly, the expression of LUG
in petals persists until at least stage 12 (Fig. 4F). In addition, LUG mRNA is found prominently in the placenta/ovules (Fig. 4G–I) and in locules of anthers (Fig. 4H). 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. 2B). 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. 5A and C), whereas GFP alone localizes to both cytoplasm and nucleus (Fig. 5B 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 LUF5S motif, and two Q-rich regions. The WD repeats, which mediate protein–protein interactions, are found in proteins with...
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 LUF5 motif is unknown, the S. cerevisiae Fló8, which also possesses the LUF5 domain near its NH2 terminus, is a transcriptional activator of Fio1 (17). However, there is no evidence that Fló8 binds to Fio1 DNA directly, and the LUF5 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 yeast Tup1, Drosophila Gro, and mammalian TLE (transducin-like enhancer of split) (Fig. 2C; 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 Fio1), and DNA damage-induced genes (27, 28). The NH2-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 a2 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 NH2 terminus and WD repeats at the COOH terminus (Fig. 2C), 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 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 provide 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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