Transcriptional repression of target genes by LEUNIG and SEUSS, two interacting regulatory proteins for Arabidopsis flower development

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Transcription repression plays important roles in preventing crucial regulatory proteins from being expressed in inappropriate temporal or spatial domains. LEUNIG (LUG) and SEUSS (SEU) normally act to prevent ectopic expression of the floral homeotic gene AGAMOUS in flowers. LUG encodes a protein with sequence similarities to the yeast Tup1 corepressor. SEU encodes a plant-specific regulatory protein with sequence similarity in a conserved dimerization domain to the LIM-domain binding 1/Chip proteins in mouse and Drosophila. Despite the molecular isolation of LUG and SEU, the biochemical function of these two proteins remains uncharacterized, and the mechanism of AGAMOUS repression remains unknown. Here, we report that LUG and SEU interact directly in vitro and in vivo. Furthermore, LUG exhibits a strong repressor activity on several heterologous promoters in yeast and plant cells. SEU, in contrast, does not exhibit any direct repressor activity, but can repress reporter gene expression only in the presence of LUG, indicating a possible role of SEU as an adaptor protein for LUG. Our results demonstrate that LUG encodes a functional homologue of Tup1 and that SEU may function similarly to Ssn6, an adaptor protein of Tup1. We have defined the LUFS domain, two central glutamine (Q)-rich domains, and a LIM domain-binding (Ldb) domain of LUG as both necessary and sufficient for the interaction with SEU and two domains of LUG as important for its repressor function. Our work provides functional insights into plant transcriptional corepressors and reveals both conservation and distinctions between plant corepressors and those of yeast and animals.

Transcription repression is emerging as a key regulatory strategy for both animals and plants to prevent crucial regulatory proteins from being expressed in inappropriate temporal or spatial domains. Repression is a dynamic process that regulates gene expression at two points. First, a gene can be repressed but primed for transcription, derepression resulting in rapid up-regulation of expression. Second, expression of an actively transcribed gene can be down-regulated rapidly. Repression occurs through two distinct yet overlapping mechanisms: the stabilization of nucleosomes on DNA to form a closed chromatin structure and the inactivation of the transcription machinery. Despite the conservation of repression mechanisms demonstrated in yeast and animals, our understanding of these mechanisms in higher plants remains limited.

In higher plants, normal floral development requires the proper expression and function of the floral homeotic gene AGAMOUS (AG). AG mRNA is normally expressed in the inner two whorls of a flower to specify stamen and carpel identity and to control floral meristem determinacy (1–3). We have previously identified two negative regulators of AG, namely LEUNIG (LUG) and SEUSS (SEU), which are required for the repression of AG transcription in the outer two whorls of a flower (4, 5). In flowers of both lug and seu mutants, AG mRNA is expressed in all four floral whorls, resulting in the ectopic formation of carpels and stamens in the outer two whorls (4, 5). Additionally, AG transcription is normally initiated at stage 3 floral meristems in lug and seu mutants. Furthermore, synergistic genetic interactions have been observed between lug and seu, resulting in a more severe degree of AG misexpression in lug seu double-mutant flowers (5). Genetic epistasis indicated that precocious and ectopic AG expression is responsible for the floral organ identity transformation and organ loss observed in lug and seu single and double mutants.

LUG encodes a nuclear localized protein with an N-terminal LUFS/LUh, Flo8, single-strand DNA-binding protein (SSDP) (LUFS) domain, two central glutamine (Q)-rich domains, and a C terminus 7-WD repeat domain (6). The LUFS domain is a protein motif present in LUG, LUH, Flo8, and Ssdp. However, the similarity between LUG/LUh and Flo8 or Ssdp is limited only to the LUFS domain. The Q-rich and WD-repeat domains of LUG are conserved in a class of transcriptional corepressors including Tup1 in yeast (Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Candida albicans), Groucho (Gro) in Drosophila, and transducin-like enhancer of split (TLE) in mammals (7–9). These corepressor proteins, collectively called the GroTLE family proteins (10), do not possess a DNA-binding motif but repress a diverse number of target genes through targeted recruitment by site-specific DNA-binding transcription factors. Whereas the Drosophila Gro directly binds to a VWPRY pentapeptide present in the C terminus of some transcription factors (11), yeast Tup1 interacts with DNA-binding factors through an adaptor protein, Ssn6 (12). The N-terminal domain of Tup1 interacts directly with Ssn6, which binds to specific transcription factors. Once recruited to a promoter, GroTLE proteins interact with chromatin modifying factors or components of the RNA polymerase II holoenzyme, leading to the silencing of target gene expression. The sequence and motif similarity between LUG and GroTLE family proteins as well as defects in AG repression in lug mutants suggest that LUG may encode a transcriptional corepressor.

In contrast, SEU encodes a plant protein with two Q-rich domains and a conserved central domain. This conserved central domain shows sequence similarity to the dimerization domain of LIM domain-binding (Ldb) family of transcriptional coregulators such as the Ldb1 in mouse and Chip in Drosophila (5, 13). Ldb proteins regulate transcription by means of direct physical interactions with LIM-homeodomain proteins (14–16). It was thought that the Ldb1/Chip cofactors homodimerize and thereby bridge two LIM-homeodomain proteins to form a tetrameric complex (17). Recently a third protein, Ssdp, was discovered to be a functional component of the complex (13, 18).

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Abbreviations: LUG, LEUNIG; SEU, SEUSS; AG, AGAMOUS; SSDP, single-strand DNA-binding protein; LUFS, LUG/LUh, Flo8, SSDP; Ldb1, LIM domain-binding 1; Q, glutamine; Gro, Groucho; TLE, transducin-like enhancer of split; TSA, trichostatin A; HDAC, histone deacetylase; MBP, maltose-binding protein; GAL4BD, DNA-binding domain of GAL4; GAL4AD, activation domain of GAL4; LUH, luciferase; β-gal, β-galactosidase.

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Interestingly, Sspd contains a N-terminal LUFS domain shown to interact with Ldb1/Chip. Hence, the Ldb1/Chip acts to bridge the interaction between Sspd and LIM-homeodomain.

Despite the sequence similarity between LUG and the GroTLE corepressors and between SEU and Ldb1/Chip, it is not known whether LUG can function as a transcriptional repressor, nor do we have any understanding of the molecular function of SEU. Furthermore, the molecular basis underlying the synergistic genetic interactions between seu and lug remains to be characterized. The similar single mutant phenotype, the strong synergistic genetic interaction between lug and seu suggest that SEU could function together with LUG as components of the same corepressor complex. SEU may function to bridge the interaction between LUG, a LUF5 domain protein, and other as-yet-unidentified DNA-binding factors. Alternatively, LUG and SEU could act in parallel and partially overlapping pathways to regulate AG transcription.

Here, we demonstrate that LUG functions as a transcriptional repressor by means of an apparently conserved eukaryotic transcription repression mechanism, demonstrating that LUG is a bona fide plant homologue of the GroTLE transcription corepressors. We also define a functional role of SEU, which does not exhibit any repressor activity, but rather, acts as an adaptor protein for LUG. In addition, we demonstrate a physical interaction between LUG and SEU, which parallels those between Sspd and Ldb1/Chip and suggests that the LUF5 domain is an evolutionary conserved protein–protein interaction domain. Together, these data provide insights into how plant corepressors interact to regulate target gene expression and help define the biochemical functions of SEU, the founding member of a plant-specific regulatory protein family. By understanding how LUG and SEU represses AG transcription we hope to shed light on general transcriptional repression mechanisms in higher plant development.

**Methods**

**Plasmid Construction.** The procedures of plasmid construction for the yeast two-hybrid assays, yeast repression assays, plant repression assays, and maltose-binding protein (MBP)- or GST-tagged proteins are described in Supporting Methods, which is published as supporting information on the PNAS web site. Primer sequences and primer pair combinations are listed in Tables 1 and 2, which are published as supporting information on the PNAS web site.

**Yeast Two-Hybrid Assays.** Yeast strain PJ69-4A (MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ) harbors three reporters HIS3, ADE2, and lacZ, each under the control of a different GAL4-responsive promoter (19). BD bait and AD prey plasmids were cotransformed into PJ69-4A according to the protocol described in www.umanitoba.ca/faculties/medicine/biochem/gietz/2HS.html, and were plated on selection medium before being incubated at 30°C for 3 days. Primary transformants were subsequently streaked onto plates selective for the reporter gene(s): histidine (+3 mM 3-amino-1,2,4-triazole) and adenine plates. White colonies grown on the selection plates were assayed for the β-galactosidase (β-gal) activities. For β-gal liquid assays, transformants were inoculated into selective liquid media and grown at 30°C until the OD600 reached ~1. Samples were analyzed in triplicate by using the Galacto Light Plus kit (Applied Biosystems) and the TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) according to the manufacturer’s instructions.

**Yeast Repression Assays.** FT5::LG312A S and FT5::JK1621 yeast strains contain integrated lacZ reporter genes with or without four LexA operator sites upstream of the CYC1 promoter, respectively (20). Yeast cells were transformed with either LexA-LUG (or LUG derivatives), LexA-SEU, or LexA-SEU+LUG. After overnight growth in liquid medium to an early log phase, cells were harvested and assayed for β-gal activity. Values were normalized to OD600.

**Repression Assays in Plant Cells.** Isolation and transfection of *Arabidopsis* mesophyll protoplasts was as described, and can be accessed at http://genetics.mgh.harvard.edu/sheenweb/protocols.reg.html. In cotransfection assays, 10 μg of reporter, 10 or 20 μg of effector constructs, and 0.1 μg of control plasmid 35S::LUC or 35S::RenillaLUC were used for each transfection. The total amount of DNA for each transfection remained constant (40 μg) by adding the appropriate amount of vector pART7 DNA that contains the 35S promoter and 3’ Ocs site (21). In cases where trichostatin-A (TSA) was used, the protoplasts were first transfected with DNA for 12 h, and then 20 μM TSA was added to the transfection reaction for an additional 12 h before the reporter assay. Luciferase assays were performed with the Promega dual-luciferase reporter assay system and the TD-20/20 luminometer. The fluorometric β-glucuronidase (GUS) assay was performed with the substrate MUG as described (22), and fluorescence was measured by using a Picofluor fluorometer (Turner Designs).

**In Vitro Pulldown.** The procedures for the purification of MBP- and GST-fusion proteins are described in Supporting Methods. Five micrograms of GST-SEU immobilized on the glutathione resin was mixed with 5 μg of MBP-fusion proteins and was incubated overnight at 4°C in 100 μl of binding buffer (50 mM KH2PO4, pH 7.5/50 mM NaCl/50 mM KCl/5 mM MgCl2/0.2% Triton X-100/1% BSA). Samples were washed five times in PBS buffer before being resuspended in 1× NuPage LDS sample buffer (Invitrogen), boiled, and then separated by 4–12% NuPage gel (Invitrogen). The retention of MBP-fusion proteins by GST-SEU was detected by Western blots with anti-MBP antibody (NEB, Beverly, MA).

**Results**

**The LUFS Domain of LUG Is Sufficient for Interaction with SEU.** To illuminate the molecular basis underlying the synergistic genetic interactions between seu and lug, a yeast two-hybrid assay was used to test whether SEU could physically interact with LUG. The LUG protein can be divided into three domains: the N-terminal LUFS domain, the central Q-rich domain, and the C-terminal 7-WD repeat domain. Full-length LUG, LUFS, LUG+Q, and Q+ WD were each fused to the DNA-binding domain of GAL4 (GAL4BD) and were tested for interaction with full-length SEU which was fused to the activation domain of GAL4 (GAL4AD). Whereas full-length LUG, LUFS, and LUFS+Q interacted with full-length SEU, Q+WD failed to interact with SEU (Fig. 1A). This finding suggested that the LUFS domain is both necessary and sufficient for interacting with SEU. Interestingly, the strongest interaction was observed between the LUFS and SEU (Fig. 1A, lane 4). Two alternative explanations could account for the difference in interaction strength between LUFS alone and other interacting LUG truncations. First, any repressor activity conferred by the full-length LUG-BD or LUFS-BD failed to interact with SEU (Fig. 1A). This finding suggested that the LUFS domain is both necessary and sufficient for interacting with SEU. Alternatively, the LUG-BD or LUFS+Q-BD may interfere with or partially block the accessibility of LUFS by SEU.

To establish whether the two-hybrid interactions between LUG and SEU represented a direct physical contact in vitro, we carried out GST affinity chromatography. Immobilized GST-SEU was incubated with MBP, MBP-LUFS, and MBP-LUFS+Q. In agreement with the two-hybrid data, GST-SEU (but not GST alone) interacted specifically with MBP-LUFS and...
MBP-LUFS-Q (Fig. 2), demonstrating a direct protein–protein interaction between LUG and SEU.

To define the domain(s) within SEU responsible for the interaction with LUG, similar two-hybrid analyses were performed with various SEU truncations. Full-length SEU as well as several SEU truncations were able to self-activate reporter gene expression when fused to the GAL4BD domain. SEU protein is divided into the following five regions: N and C, N- and C-terminal domains; Q1 and Q2, the two Q-rich domains; D domain, the highly conserved dimerization domain.

LUG Possesses Repressor Activities. Based on genetic and structural data, we predicted that LUG would function as a transcription repressor. By using an in vivo yeast transcription repression assay, we tested the ability of LUG or SEU to repress transcription. Full-length LUG cDNA and four truncated LUG derivatives were fused in-frame and downstream of the bacterial LexA DNA-binding domain and tested for their ability to repress lacZ expression (Fig. 3A). When directly recruited to the test promoter, LexA-LUG reduced reporter gene expression by 45% compared with LexA alone, demonstrating that LUG has a repressor activity, and suggesting that LUG can repress transcription through a conserved eukaryotic repression mechanism.

To determine whether the observed repression activity could be attributed to specific domains within the LUG protein,
truncated LUG derivatives were assayed. Two distinct domains were found to have repression function. The LUFS+Q repressed transcription to the same extent as full-length LUG (Fig. 3A), whereas the Q+WD repressed transcription to a lesser but significant extent. This repression function cannot be attributed solely to the Q-rich domain because the Q-rich domain alone cannot repress transcription.

To test whether LUG could function as a transcription repressor in planta, a transient Arabidopsis protoplast repression assay using the 2XUAS<sub>Gal4</sub>-icup::GUS reporter (23) was adopted. A 2XUAS<sub>Gal4</sub> element is located immediately upstream of the icup, a constitutive tobacco promoter, driving the GUS reporter gene. Arabidopsis leaf protoplasts were transfected with 35S::LUG-BD or 35S::SEU-BD together with the reporter, and the effects on GUS expression were quantified (Fig. 3B). Whereas SEU-BD did not show any effect on the level of GUS expression, LUG-BD significantly reduced the GUS expression level in a concentration-dependent manner. Specifically, doubling the amount of LUG-BD plasmid DNA used in the transfection resulted in an additional 3-fold reduction of GUS reporter activity (Fig. 3B). The reduced GUS activity is unlikely due to simple steric interference of the transcription machinery by increased LUG-BD at the promoter region because a similar increase in SEU-BD DNA did not result in any decrease in GUS expression (Fig. 3B). Clearly, LUG functions as a strong repressor of transcription in planta and can repress transcription more efficiently in a homologous system than the heterologous yeast system.

LUG Is Required by SEU to Repress Transcription in Yeast and in Planta. In contrast to LUG, the protein sequence of SEU provided scant clues as to the biochemical function of SEU. SEU was unable to repress transcription when fused to the LexA in yeast repression assays (Fig. 4A). However, when LUG was overexpressed together with LexA-SEU, a similar level of repression to LexA-LUG was observed (Fig. 4A), indicating a functional molecular interaction between these two proteins. What then is the biological significance of the SEU–LUG interaction? One possibility as suggested by the above yeast repression assay is that SEU may serve as an adaptor protein facilitating the interaction between LUG and DNA-binding transcription factors just as the yeast corepressor Ssn6 and the mouse and Drosophila Ldb1/Chip proteins. To assess this possibility in planta, Arabidopsis protoplasts were cotransfected with a reporter and 35S::SEU-BD in the absence or presence of 35S::LUG (or various 35S::LUG truncations; Fig. 4B). The reporter, 2XUAS<sub>Gal4</sub>-35S::LUC, contains the luciferase (LUC) gene under the control of a constitutive 35S promoter with 2XUAS<sub>Gal4</sub> located immediately upstream of the 35S promoter. The use of 35S::SEU-BD alone had no effect on the reporter activity (Fig. 4B). Yeast repression assays using reporters described in Fig. 3A. LexA-SEU (full-length), LexA-LUG (full-length), as well as LexA-SEU+LUG are tested for their repressor activities. Whereas LexA-SEU alone showed no repressor activity, cotransfection of LexA-SEU and LUG together showed repressor activity. (B) Transient Arabidopsis protoplast repression assays using reporter 2XUAS-35S::LUC mixed with 35S::RenillaLUC (35S::LUC-R). LUC/LUC-R ratio was used to indicate reporter gene expression and to control for transfection efficiency. Ten micrograms of GAL4BD DNA or 35S::SEU-BD DNA was mixed with (or without) 20 µg of DNA of various 35S::LUG derivatives. In lanes 5, 7, and 9, 20 µM TSA was used. (Bar, SD.)

Fig. 3. LUG represses reporter gene expression in yeast and plant cells when tethered to test promoters. (A) Yeast repression assays with a lacZ reporter containing LexA operator sites (lop) upstream of the CYC1<sub>UAS</sub>-CYC1<sub>TATA</sub> promoter integrated into the yeast genome. Yeast cells were independently transformed with the indicated LexA fusion proteins, and β-gal activities (average of five independent transformants) are shown. No effect was seen with reporters lacking lop. LexA alone is a negative control. LexA-Ssn6 serves as a positive control. LUFS, Q, LUFS+Q, and Q+WD are four different LUG truncations each fused to the LexA DNA-binding domain; LUG indicates the full-length LUG fused to LexA. (B) Transient plant repression assays. 2XUAS-icup::GUS reporter plasmid was mixed with 35S::LUC plasmid and was transfected into Arabidopsis protoplasts. GUS/LUC ratio equalizes differences in transfection efficiency. Different effector DNAs were introduced simultaneously with the reporter DNA. pART7 vector alone; SEU-BD, GAL4BD fused to full-length SEU; LUG-BD, GAL4BD fused to full-length LUG. All effector proteins were expressed from the CaMV 35S promoter. 1× and 2× indicate equal (10 µg) and twice (20 µg) the amount of effector DNA compared with the 2XUAS-icup::GUS reporter DNA. (Bar, SD.)

Fig. 4. SEU acts as an adaptor protein for LUG. (A) Yeast repression assays using reporters described in Fig. 3A. LexA-SEU (full-length), LexA-LUG (full-length), as well as LexA-SEU+LUG are tested for their repressor activities. Whereas LexA-SEU alone showed no repressor activity, cotransfection of LexA-SEU and LUG together showed repressor activity. (B) Transient Arabidopsis protoplast repression assays using reporter 2XUAS-35S::LUC mixed with 35S::RenillaLUC (35S::LUC-R). LUC/LUC-R ratio was used to indicate reporter gene expression and to control for transfection efficiency. Ten micrograms of GAL4BD DNA or 35S::SEU-BD DNA was mixed with (or without) 20 µg of DNA of various 35S::LUG derivatives. In lanes 5, 7, and 9, 20 µM TSA was used. (Bar, SD.)

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Whereas the LUFS domain of LUG interacts specifically with SEU, our in vivo and in vitro data demonstrate that the entire SEU protein is required for LUG–SEU interaction. SEU contains a Ldb1/Chip conserved domain (LCCD), a stretch of 49 highly conserved residues located C-terminal to the dimerization domain, which is essential for the interaction of Chip or Ldb1 with the LUFS domain of Ssdp proteins from Drosophila to mice (13). Although this LCCD domain is highly conserved in SEU, our study showed that the interaction with LUFS appears dependent on multiple regions of SEU, which may include, but are not limited to, the LCCD domain.

**SEU May Function as an Adaptor Protein for LUG.** Despite the sequence similarities between SEU and Ldb1/Chip in the dimerization domain, our results suggest that the genetic synergy observed between lug and seu is due to the disruption of the functional components of this plant corepressor complex. Specifically, the LUFS domain of LUG is both necessary and sufficient for interaction with SEU. The LUFS domain was originally identified and named by us, based on the high degree of conservation of this domain between LUG and LUH in Arabidopsis, Flo8 in yeast, and Ssdp in humans (6). The first half of the LUFS domain exhibits significant sequence similarity to the lissencephaly type-1-like homology (LisH) domain found in numerous animal, fungi, and plant proteins. It has been suggested that the LisH domain may be involved in mediating protein dimerization (27, 28). The second half of the LUFS domain comprises of a core amino acid sequence motif P-X-GFX-XX-WW-X-VFWD (13). Hence, the LUFS domain represents an evolutionarily conserved protein interface for transcriptional regulation in plants as well as in animals.
ization and the LCCD domains, both the N- and C-terminal regions flanking these domains are unique to SEU. In addition, SEU does not encode the LIM-interaction-domain that is essential for Ldb1/Chip’s interaction with LIM-homodomain. SEU therefore defines a class of plant-specific transcription factors and is a member of a small gene family in Arabidopsis (5), the molecular function of which is largely unknown. The results presented here indicate that SEU does not have any inherent function in repressing transcription and, on the contrary, may have an intrinsic activation potential as revealed in the yeast two-hybrid assays. Additionally, despite the presence of endogenous expression of LUG, 355::SEU-BD alone failed to repress reporter gene expression in the Arabidopsis protoplasts (likely due to a low level of endogenous LUG expression in Arabidopsis leaves; ref. 6). Only simultaneous transfection of SEU-BD and LUG gave measurable repressor activities. This situation is analogous to the Snf6-Tup1 interaction in yeast, where Snf6 has an absolute requirement for Tup1 to repress transcription, and Snf6 was shown to activate transcription in the absence of Tup1 (29). Hence, SEU appears to be a functional homologue of Snf6, despite the similarity between SEU and Snf6 proteins being limited to the presence of Q-rich domains.

Whether the apparent functional homology between SEU and Snf6 extends to SEU interacting with specific DNA-binding transcription factors remains to be determined. Preliminary yeast two-hybrid assays failed to detect an interaction between LUG and any of the tested floral regulatory genes (V.V.S., A.S., Z.L., unpublished data) including APETALA2, AINTEGMENTA, APETALA1, SEPALLATA3, BELLRINGER, and LEAFY (30, 31). However, a similar yeast two-hybrid assay used to detect interactions between SEU and the above floral regulatory factors revealed an interaction between SEU and APETALA1, and between SEU and SEPALLATA3 (V.V.S., A.S., and Z.L., unpublished data). Nevertheless, the biological significance of such interactions remains to be established. Recently, a role of SEU in auxin response was revealed (32), and both genetic and direct physical interactions between SEU and the ARF3/ETTIN genes were detected, suggesting that SEU could interact with the DNA-binding transcription factor ARF3/ETTIN to regulate the expression of auxin-responsive genes.

An important function of the Ssdp and Ldb1/Chip interaction is the nuclear import of Ssdp, which lacks a nuclear localization signal. In Drosophila Chip mutants, Ssdp remains in the cytoplasm (13). We have eliminated this possible function for the SEU–LUG interaction. Hence, while not having an intrinsic transcription repressor activity, the main function of SEU appears to be an adaptor protein recruiting LUG to target promoters. Our findings demonstrate for the first time, to our knowledge, that plant corepressors function through a mechanism similar to yeast and animal corepressors and underpin future research exploring the molecular mechanisms used by corepressors in plants.

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