LEUNIG_HOMOLOG and *LEUNIG* Perform Partially Redundant Functions during Arabidopsis Embryo and Floral Development[1][C][W][OA]

Jayashree Sitaraman[2], Minh Bui[2], and Zhongchi Liu*

Department of Cell Biology and Molecular Genetics (J.S., M.B., Z.L.), and Department of Biology Graduate Program (M.B.), University of Maryland, College Park, Maryland 20742

Transcription corepressors play important roles in animal and plant development. In Arabidopsis (*Arabidopsis thaliana*), *LEUNIG* (*LUG*) and *LEUNIG_HOMOLOG* (*LUH*) encode two highly homologous proteins that are similar to the animal and fungal Gro/Tup1-type corepressors. LUG was previously shown to form a putative corepressor complex with another protein, SEUSS (SEU), and to repress the transcription of *AGAMOUS* in floral organ identity specification. However, the function of *LUH* is completely unknown. Here, we show that single *luh* loss-of-function mutants develop normal flowers, but *lug; luh* double mutants are embryo lethal, uncovering a previously unknown function of *LUG* and *LUH* in embryonic development. In addition, *luh*/+ enhances the floral phenotype of *lug*, revealing a minor role of *LUH* in flower development. Functional diversification between *LUH* and *LUG* is evidenced by the inability of *35S::LUH* overexpression to rescue *lug* mutants and by the opposite expression trends of *LUG* and *LUH* in response to biotic and abiotic stresses. The *luh-1* mutation does not enhance the defect of *seu* in flower development, but LUH could directly interact with SEU in yeast. We propose a model that explains the complex relationships among LUH, LUG, and SEU. As most eukaryotes have undergone at least one round of whole-genome duplication during evolution, gene duplication and functional diversification are important issues to consider in uncovering gene function. Our study provides important insights into the complexity in the relationship between two highly homologous paralogous genes.

Transcription repression plays a key regulatory role in cell fate specification, hormone signaling, and plant stress responses. *LEUNIG* (*LUG*) was first identified in Arabidopsis (*Arabidopsis thaliana*) based on its role in regulating the stage- and domain-specific expression of the C class floral homeotic gene *AGAMOUS* (*AG*) in flower development (Liu and Meyerowitz, 1995). In *lug* mutants, ectopic *AG* expression in the outer two whorls of a flower leads to homeotic transformation of sepals into carpels and petals into stamens as well as a reduction of floral organs. LUG protein is similar in domain structure and biochemical function to the Groucho (Gro), Transducin-Like Enhancer of Split, and Tup1 family corepressors in *Drosophila*, mammals, and yeast, respectively (Liu and Karmarkar, 2008). These corepressors do not possess a DNA-binding domain and are recruited to their regulatory targets by interacting with DNA-bound transcription factors.

The N terminus of LUG possesses a conserved domain, the LUFS domain, named after the four founding members LUG, LUH (for LEUNIG_HOMOLOG), yeast Flo8, and human SSDP (for single-stranded DNA-binding protein). The LUFS domain of LUG is essential for the direct interaction with its cofactor SEUSS (SEU; Sridhar et al., 2004). *SEU* encodes a Gln (Q)-rich protein with a centrally positioned dimerization domain also present in the LIM domain-binding family of transcriptional coregulators in mammals and *Drosophila* (Franks et al., 2002). Recruitment of the LUG/SEU corepressor complex by the MADS box proteins APETALA1 (AP1) and SEPALLATA3 (SEP3) was shown to target the LUG/SEU corepressors to the *AG* cis-regulatory element, leading to repressed chromatin at the *AG* locus (Sridhar et al., 2006). The repressor activity of LUG was shown to depend on histone deacetylase (HDAC) activity, and LUG was shown to directly interact with HDAC19 (Sridhar et al., 2004; Gonzalez et al., 2007), suggesting that the plant Gro/Tup1 family corepressors mediate transcription repression by histone modification and chromatin reorganization. Recently, LUG was shown to repress gene expression via a HDAC-independent but mediator-dependent mechanism (Gonzalez et al., 2007).

Like corepressors in animals and fungi, *LUG/SEU* possesses multiple functions. *lug* mutants showed defects in gynoecium development, female and male

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[2] These authors contributed equally to the article.

* Corresponding author; e-mail zliu@umd.edu.

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fertility, leaf and floral organ shape, and vasculature (Liu and Meyerowitz, 1995; Chen et al., 2000; Liu et al., 2000; Cnops et al., 2004; Franks et al., 2006). *Antirrhinum* mutants of *STYLOSA*, a *LUG* ortholog, not only showed abnormal flower development but also exhibited hypersensitivity toward auxin and polar auxin inhibitors (Navarro et al., 2004). A transcriptome study identified *LUG*-regulated genes in abiotic and biotic stress response, meristem function, and transport (Gonzalez et al., 2007). Therefore, *LUG* likely encodes a global regulator for multiple developmental processes and signal pathways.

In Arabidopsis, *LUG* belongs to a small family of about 13 genes (http://smart.embl-heidelberg.de/), including *TOPLESS* (*TPL*), *TOPLESS-RELATED*, and *WUSCHEL-INTERACTING PROTEIN*s (*WSIP*s; Kieffer et al., 2006; Long et al., 2006; Liu and Karmarkar, 2008). These genes are involved in regulating embryonic shoot-root axis determination and appear to repress auxin-mediated signaling events during embryogenesis (Szemenyei et al., 2008). The *TPL/WSIP* genes are also involved in mediating the effect of *WUSCHEL* in target gene repression to maintain the stem cell pool at the shoot apical meristem (Kieffer et al., 2006). Therefore, the plant Gro/Tup1 family corepressors are emerging as a fundamentally important class of regulators in plant development.

Among the 13 Arabidopsis Gro/Tup1 corepressor-like proteins, LUH (At2g32700) is most similar to LUG (Conner and Liu, 2000). Both proteins possess an N-terminal LUFS domain that is 80% identical (Fig. 1). In addition, both proteins possess seven WD repeats at the C terminus that show 58% identity to each other. A third domain that immediately precedes the WD repeats (residues 369–500) also shows a high level of sequence similarity (57%). Both LUG and LUH have centrally located Q-rich regions, but the Q-rich regions in LUH are less continuous and less extensive than those in LUG.

Despite the significant sequence similarity, almost nothing is known about *LUH* function or expression. This article presents data indicating that *LUH* possesses both unique and overlapping functions with *LUG* and that *LUH* activity is required for proper embryo and flower development. We propose a model that explains the complex relationship between *LUH* and *LUG*.

RESULTS

luh-1 Mutants Exhibit Vegetative Defects

Through the Arabidopsis TILLING Project (McCallum et al., 2000), we obtained several *luh* mutations (see ''Materials and Methods''). *luh-1* (*luh*_172H3) is caused by a G-to-A change resulting in the conversion of Trp (W-55) to a STOP codon (Fig. 1; Supplemental Fig. S1), truncating the protein at the 55th residue. *luh-2* (*luh*_147A6) changes C to T, resulting in an amino acid substitution from Ser (S-123) to Phe (F). *luh-3* (SALK_107245C) is caused by a T-DNA insertion in the third to the last exon (Supplemental Fig. S1), disrupting the last three WD repeats. We have focused on *luh-1* as it likely represents a null or a strong loss-of-function mutation.

luh-1 single mutants did not exhibit any abnormality in flowers (Fig. 2, A and B). Nevertheless, *luh-1* mutant seedlings showed slower and poorer germination on Murashige and Skoog (MS) medium (Fig. 2, C and D), with a germination rate of about 80% of wild type (Fig. 2H). In addition, *luh-1* mutants grew slower compared with the wild type (Fig. 2E) but eventually caught up. Finally, the roots of *luh-1* seedlings were significantly shorter than wild-type roots (Fig. 2, F and G). To test whether these phenotypes are caused by the *luh-1* mutation, *35S::LUH* cDNA was transformed into *luh-1* mutants. Eight transgenic plants were obtained, and two of these transgenic lines (lines 4 and 5) showed a higher level of *LUH* mRNA (Fig. 2I) and were further analyzed. The developmental defects of *luh-1* described above were rescued by the *35S::LUH* transgene. Figure 2, G and H, illustrate the rescue of root length and germination rate, respectively.

luh-1/+ Enhances Defects of *lug* in Flowers

It is possible that the function of *LUH* in flowers is not necessary when *LUG* is intact but becomes necessary when *LUG* is absent or reduced. If this is the case, *luh-1* may enhance the phenotype of *lug*. To test this, both the weak *lug-16* and the strong *lug-3* were crossed into *luh-1* to construct *lug-16; luh-1* and *lug-3; luh-1* double mutants. F2 progeny segregated *lug* single mutants as well as mutants with a more severe phenotype than *lug* single mutants. Allele-discriminating single nucleotide polymorphism (SNP) assays (see ''Materials and Methods'') were used to genotype F2 plants with a more severe phenotype than *lug* single mutants, and they were found to be homozygous for *lug* and heterozygous for *luh-1*. Specifically, while *lug-16* single mutants developed elongated siliques (Fig. 3A), the *lug-16/lug-16; luh-1/+* plants did not show any silique elongation and were completely sterile (Fig. 3B). However, the floral phenotype of *lug-16/lug-16; luh-1/+* was similar to that of *lug-16* single mutants (Fig. 3, C and D).

lug-3/lug-3; luh-1/+ flowers exhibited a more severe phenotype than *lug-3* single mutant flowers (Fig. 3, E and F). *lug-3/lug-3; luh-1/+* mutant flowers consisted of only a few carpelloid sepals and sepal-like organs topped with horns, suggesting a more severe homeotic transformation, possibly caused by more extensive ectopic expression of *AG*. There was no petal, and stamens were either completely absent or partially fused to first whorl organs (Fig. 3F). The *lug-3/lug-3; luh-1/+* flowers are completely sterile, and they resemble *lug; seu* double mutant flowers (Franks et al., 2002).

Among the F2 progeny of the *lug-3* and *luh-1* cross, *lug-3; luh-1* double mutants were never found, although

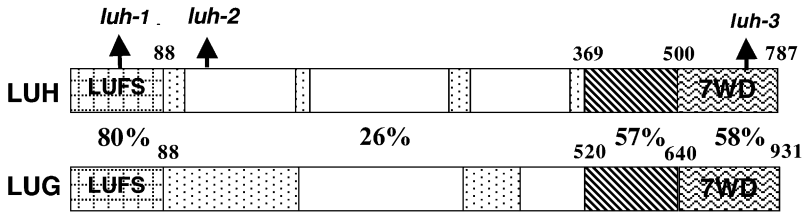

Figure 1. Schematic diagram showing the protein domains of LUH and LUG. Numbers represent amino acid positions, and percentage values indicate the percentage identity between LUH and LUG. In addition to the N-terminal LUFS domain and the C-terminal seven WD repeats, a region immediately preceding the WD repeats (box with diagonal lines) is also highly conserved. The dotted boxes represent Q-rich regions. The location of each *luh* allele is indicated by an arrow.

they should occur at a frequency of one in 16. Only two *lug-16; luh-1* double homozygous mutants were found after screening several hundred F2 progeny of the *lug-16* and *luh-1* cross. The *lug-16; luh-1* double mutants were extremely small in stature; the entire mature plant is smaller than a single rosette leaf (Fig. 3G). The inflorescence meristem only bears three to five flowers consisting of only carpels (Fig. 3H).

Since SEU acts as an adaptor for LUG and the *seu* mutation enhances *lug* mutants (Franks et al., 2002; Sridhar et al., 2004), we tested the genetic interaction between *luh* and *seu*. *seu-1; luh-1* double mutants were identified by genotyping F2 as well as F3 plants of the *seu-1* and *luh-1* cross. The *seu-1; luh-1* double mutants are morphologically indistinguishable from *seu-1* single mutants (Fig. 3, I and J).

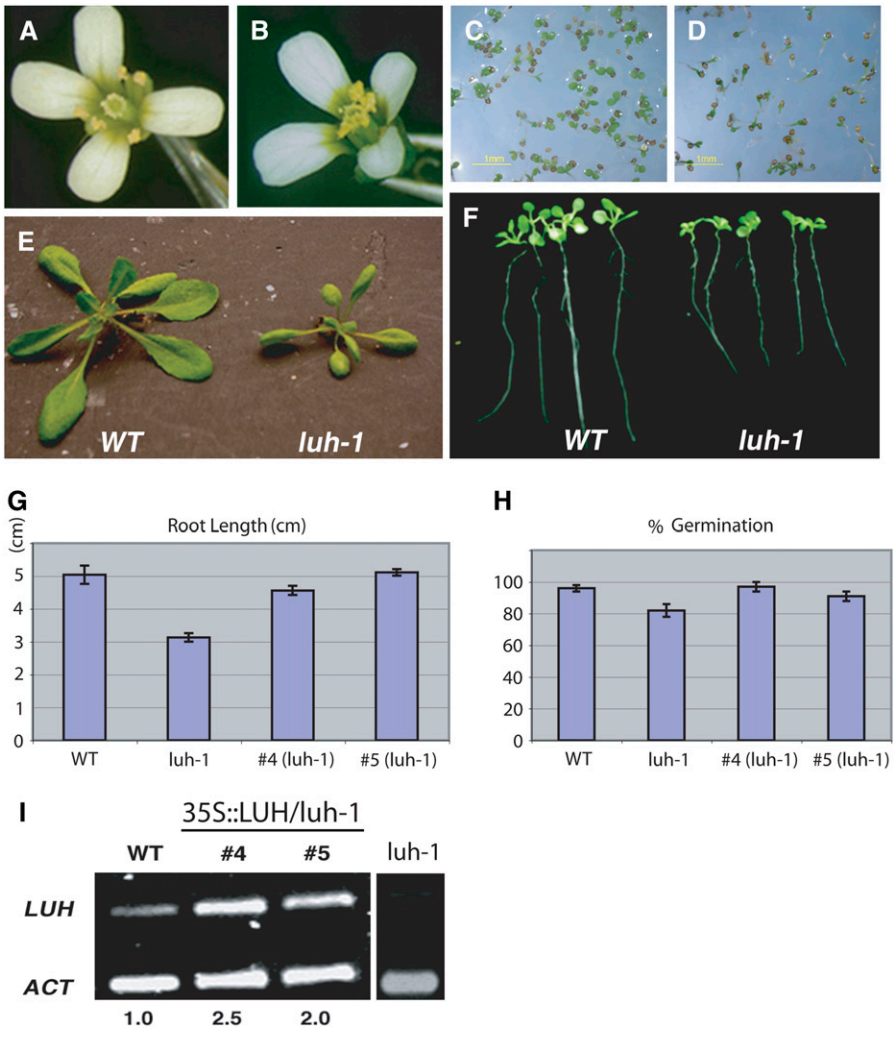

Figure 2. *luh-1* develops normal flowers but exhibits defects in vegetative growth. A, A wild-type (Columbia *erecta*-105) flower. B, A *luh-1* flower in the Columbia *erecta*-105 background. C, Germination of wild-type seeds on MS medium at 5 d. D, Germination of *luh-1* seeds on MS medium at 5 d. E, Three-week-old wild-type and *luh-1* plants. F, Root elongation of wild-type and *luh-1* seedlings on MS medium. Germinated seedlings were transferred to MS plates and grown on vertical plates. Photographs were taken after 7 d. G, *35S::LUH* complemented the *luh-1* defect in root elongation as shown in transgenic lines 4 and 5. Root length, measured after 7 d, was expressed as mean ± SE. H, Germination phenotype of *luh-1* is complemented by *35S::LUH*. Germination is expressed as mean ± SE. I, Semiquantitative RT-PCR showing the expression of *LUH* mRNA in the wild type (Columbia *erecta*-105), *luh-1*, and two different *35S::LUH* transgenic lines (lines 4 and 5) in the *luh-1* background. The ratio between *LUH* band intensity and that of the *ACT2* control band, which is set to 1 in wild type, is shown below each lane.

Most *luh; lug* Double Mutants Are Embryo Lethal

The absence of *lug-3; luh-1* double mutants and a significant reduction of *lug-16; luh-1* double mutants among the F2 progeny suggested that most *lug; luh-1* double mutants die prematurely. The complete sterility of *lug-16/lug-16; luh-1/+* plants (Fig. 3, A and B) makes it impossible to identify *lug-16; luh-1* double mutants in the next generation. Instead, we identified several *luh-1/luh-1; lug-16/+* plants through genotyping F2 progeny of the *lug-16* and *luh-1* cross. Surprisingly, these *luh-1/luh-1; lug-16/+* plants developed wild-type-like flowers, albeit at a slightly smaller size (Fig. 4, A and D). Thus, it appears that *LUG* is more critical for proper flower development than *LUH*, as *luh-1/luh-1; lug-16/+* plants with only one copy of wild-type *LUG* are capable of normal floral development but *lug-16/lug-16; luh-1/+* plants with only one copy of wild-type *LUH* fail to develop normal flowers (Fig. 3).

When *luh-1/luh-1; lug-16/+* plants were self-fertilized and their siliques were examined, white and abnormal seeds occurred at a frequency of about 36% in a silique (Fig. 4E; Supplemental Table S1). This is in contrast to *luh-1* and *lug-16* single mutants, whose siliques contain only about 5% of white seeds (Fig. 4, B and C; Supplemental Table S1). To verify the genotype of white and green seeds segregated by *luh-1/luh-1; lug-16/+* plants, eight white seeds and 10 greens seeds (collected from several different siliques) were individually genotyped. All eight white seeds were found to be *luh-1/luh-1; lug-16/+*. Among the 10 green seeds, six were *luh-1/luh-1; +/+* and four were *luh-1/luh-1; lug-16/+*. This suggests that *luh-1/luh-1; lug-16/+* seeds could develop into either normal green seeds or abnormal white seeds. An absence of *luh-1/luh-1; lug-16/lug-16* genotype among the eight white and 10 green seeds indicated that the *luh-1/luh-1; lug-16/lug-16* embryos died early during embryogenesis, before visible seeds were formed. Therefore, significant functional redundancy must exist between *LUH* and *LUG* during early embryo development.

To better pinpoint the stage at which embryo development is affected in the white seeds, we examined the white and green seeds dissected from the same siliques of *luh-1/luh-1; lug-16/+* plants. While the green embryos were already at the torpedo stage (Fig. 4F), the white embryos from the same silique were arrested at the late globular stage (Fig. 4G). In some of the white seeds, the globular embryos appeared disintegrated (data not shown).

LUH and *LUG* Exhibit Divergent Functions and Expression Patterns

Functional diversification between *LUH* and *LUG* could result from their differences in expression or in protein-coding sequences or both. To test this, we transformed *35S::LUH* into *lug-16* mutants. *lug-16* is the most fertile allele and can be easily transformed. If overexpressing *LUH* could rescue *lug-16*, the *LUH* coding region may be equivalent to that of *LUG*. None of the 12 T1 transformants was able to rescue *lug-16*. On the contrary, five of these 12 lines showed an enhanced phenotype, with more carpelloid sepals and a greatly reduced organ number (Fig. 5, C and D). We hypothesized that these five lines exhibited cosuppression and silencing of the endogenous *LUH*. Semiquantitative reverse transcription (RT)-PCR was performed for three of the five lines, revealing that that the *LUH* mRNA level in these lines was approximately half of that in wild-type plants (Fig. 5E) and supporting the cosuppression hypothesis.

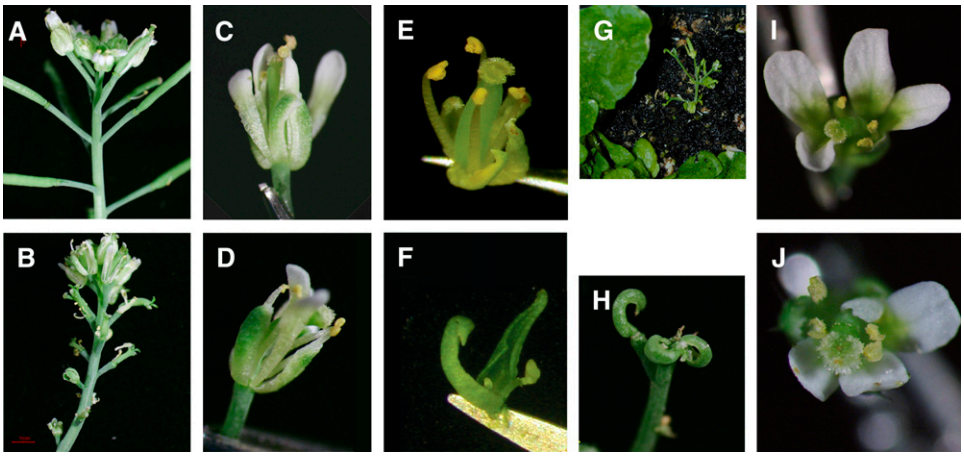

Figure 3. *luh-1* enhances *lug-16* and *lug-3* during flower development. A, An inflorescence shoot of *lug-16*. Note the elongating siliques. B, An inflorescence shoot of *lug-16/lug-16; luh-1/+*. Note the absence of silique development. C, A *lug-16* flower with narrow sepals and petals. D, A *lug-16/lug-16; luh-1/+* flower. E, A *lug-3* flower. F, A *lug-3/lug-3; luh-1/+* flower. G, A *lug-16; luh-1* double mutant. Note the extreme small stature; the mature plant is smaller than a rosette leaf. H, A close-up of an inflorescence shoot of the *lug-16; luh-1* double mutant. I, A *seu-1* flower. Note the reduced stamen number. J, A *seu-1; luh-1* double mutant flower.

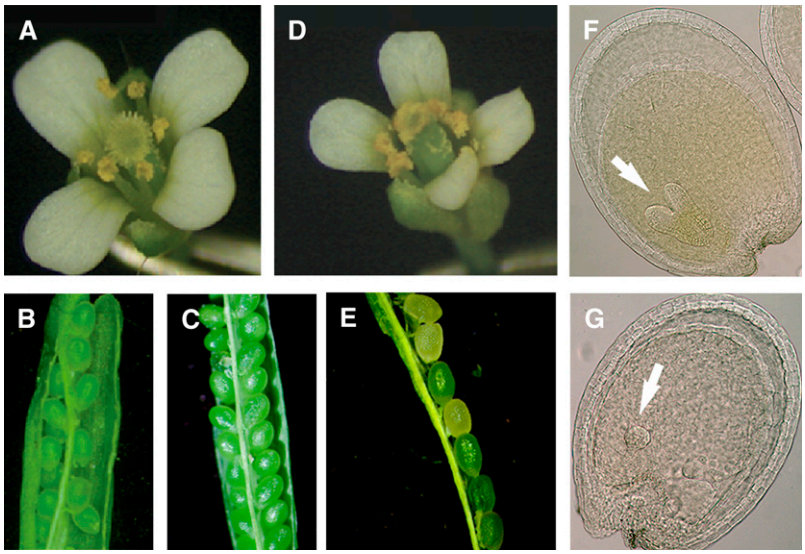

Figure 4. *luh-1; lug-16* double mutants are embryo lethal. A, *luh-1* flower. B, An open silique of *luh-1* showing green seeds inside. C, An open silique of *lug-16* showing green seeds inside. D, A *luh-1/luh-1; lug-16/+* flower. Note the smaller flower size. E, An open *luh-1/luh-1; lug-16/+* silique showing white seeds among green seeds. F, Nomarski image of a green seed in a silique derived from a *luh-1/luh-1; lug-16/+* plant. The arrow indicates the embryo proper at the torpedo stage. G, Nomarski image of a white seed from the same silique as F. The arrow indicates an embryo at the late globular stage.

The remaining seven *35S::LUH*; *lug-16* lines did not show a cosuppressed phenotype, but the *35S::LUH* transgene did not rescue *lug-16* (data not shown). Either they did not express high enough levels of *LUH* or LUH protein is not equivalent to LUG in function. To distinguish these alternative explanations, *35S::LUH*; *luh-1* transgenic line 5, previously shown to rescue *luh-1* phenotype (Fig. 2), was crossed into *lug-16*. The F2 plants harboring *35S::LUH* line 5 and carrying wild-type *LUH* and *LUG* are wild type in phenotype (Fig. 5, F and G). However, the F2 *35S::LUH* line 5 plants carrying the wild-type *LUH* allele but the *lug-16/lug-16* mutant allele exhibited phenotypes identical to the *lug-16* single mutants (Fig. 5, H and I), suggesting that increasing (and ectopic expressing) *LUH* transcripts could not substitute for *LUG*.

To compare the expression patterns of LUG and LUH during development, we utilized the AtGenExpress atlas that compares the expression profiles of 22,746 probe sets on the Affymetrix ATH1 array using triplicate expression estimates from 79 diverse development samples ranging from embryogenesis to senescence and from roots to flowers (Schmid et al., 2005). *LUG* and *LUH* were shown to be expressed in all

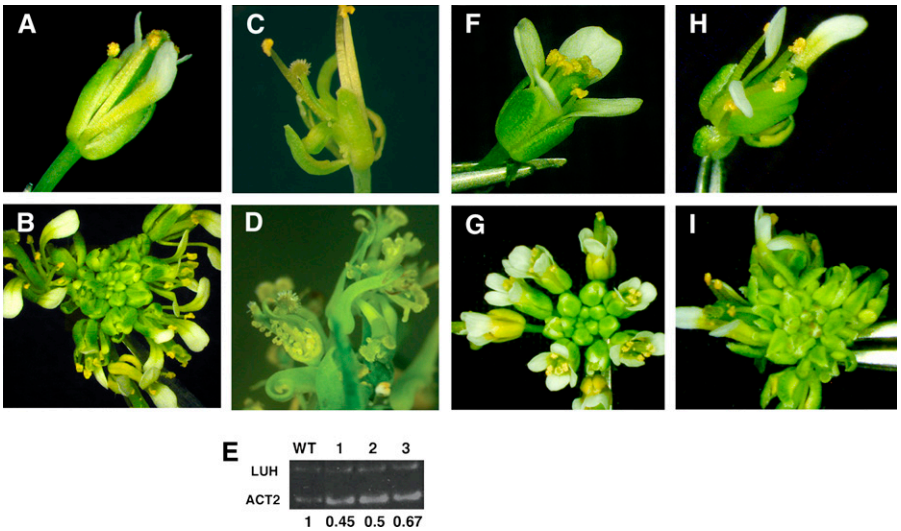

Figure 5. *35S::LUH* failed to rescue *lug-16* mutants. A, A *lug-16* mutant flower. B, A *lug-16* inflorescence. C, A *35S::LUH; lug-16* flower, where the *35S::LUH* appears to enhance the defect of *lug-16*, probably by silencing endogenous *LUH*. D, An inflorescence of a *35S::LUH; lug-16* transgenic plant similar to that in C. E, RT-PCR result showing reduced *LUH* mRNA in the *35S::LUH; lug-16* transgenic lines 1, 2, and 3. The numbers below represent the relative mRNA level normalized to *ACT2* and compared with the wild type, which is taken as 1. F and G, *35S::LUH* line 5 in the wild type, causing no obvious phenotype. H and I, *35S::LUH* line 5 in *lug-16*, showing phenotypes identical to those of the *lug-16* mutants shown in A and B. [See online article for color version of this figure.]

79 samples at comparable levels (Fig. 6A). Interestingly, *SEU*, the partner of *LUG*, showed an almost identical expression profile to *LUG*, supporting that proteins present in the same complex are likely expressed in similar profiles (Schmid et al., 2005). A comparison between *LUH* and *SEU* revealed highly similar but not identical profiles (Fig. 6A).

In addition, the expression profiles of *LUG*, *SEU*, and *LUH* were compared using the AtGenExpress data with Arabidopsis samples challenged with biotic and abiotic stresses, hormones, lights, and nutrients (www.weigelworld.org/resources/microarray/AtGenExpress/; Kilian et al., 2007). As shown in Figure 6B, an increased expression upon a particular treatment is indicated by magenta and a decreased expression upon a treatment is indicated by green. The clustergram showed that *LUH* and *LUG* exhibited almost opposite expression trends upon treatment with similar conditions. For example, *LUH* transcription is induced by exposures to biotic stress (nematode and *Botrytis cinerea*) and abiotic stress (salt, genotoxic, wounding, drought, oxidative); *LUG* transcription, on the contrary, is reduced or unchanged under these same conditions. Additionally, certain chemicals (cycloheximide, 2,4-dichlorophenoxyacetic acid, $AgNO_3$, aminoethoxyvinylglycine), biotic stress (*Agrobacterium tumefaciens*), and abiotic stress (hypoxia) caused increased *LUG* expression but reduced *LUH* expression. *SEU* appears to exhibit an expression pattern more similar to that of *LUH*. This analysis suggests that *LUG* and *LUH* are

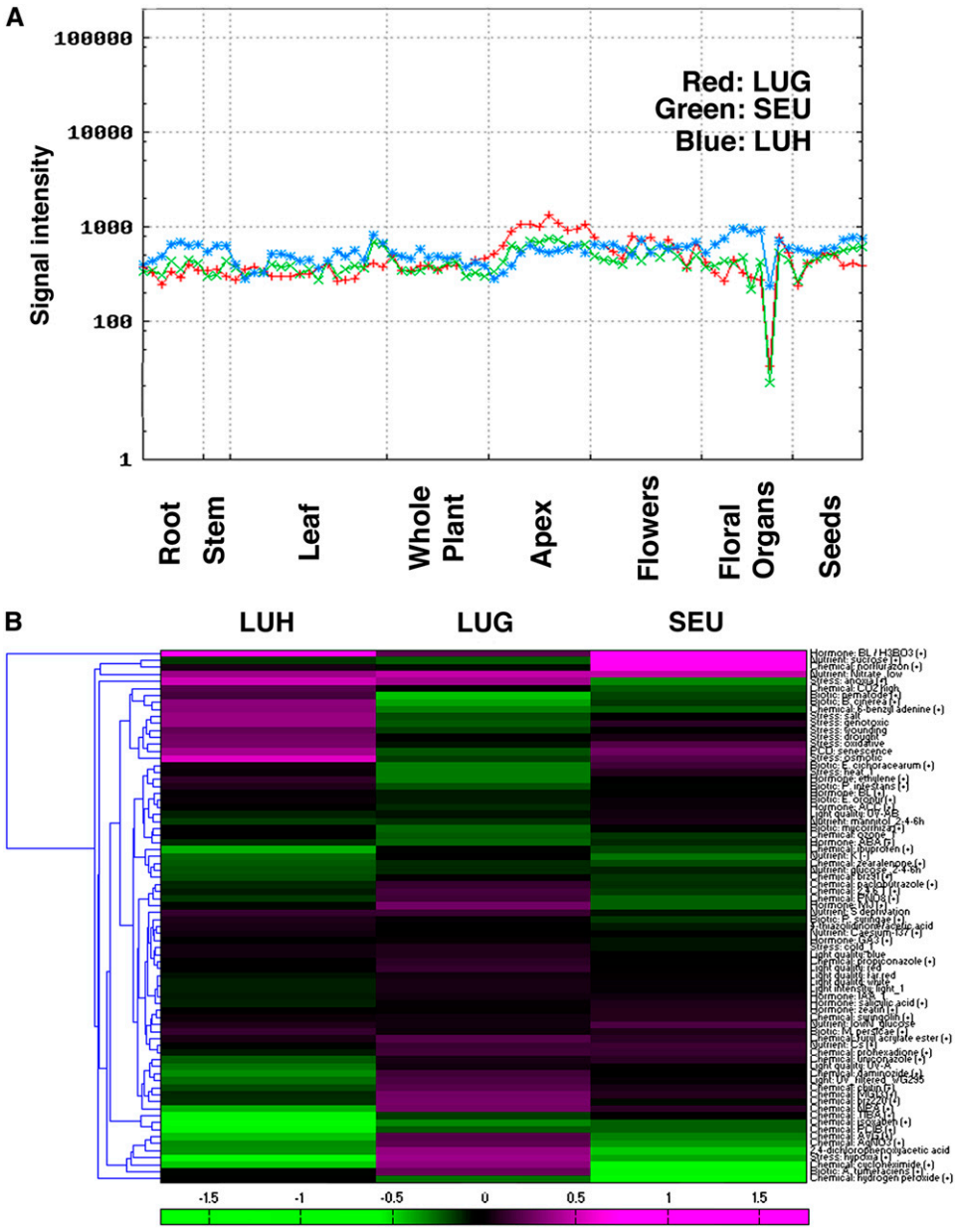

Figure 6. *LUH* expression in comparison with *LUG* and *SEU*. A, Expression profile of *LUH*, *LUG*, and *SEU* in different developmental tissues and stages. The data were generated by AtGenExpress (Development) and presented using the AtGenExpression Visualization Tool (Schmid et al., 2005). *LUG* (red) and *SEU* (green) showed almost complete coexpression in all tissues. *LUH* (blue) closely resembled but was not identical to the *LUG*/*SEU* profile. B, Hierarchal cluster analysis of environmental regulation of *LUH*, *LUG*, and *SEU* expression using AtGenExpression (Abiotic, Light, Hormone, Pathogen) estimates by gcRMA (Kilian et al., 2007; www.weigelworld.org/resources/microarray/AtGenExpress/). The clustergram was generated with the Matlab RC13 (Mathworks) Bioinformatics Toolbox. An increase in expression is indicated by magenta, and a decrease in expression is indicated by green (see bar at bottom).

utilized to play opposite regulatory roles in different stress signal pathways.

LUH Interacts Directly with SEU But Not with LUG

If *LUH* and *LUG* have both overlapping and unique functions, do their proteins interact directly with each other to form heterodimers? Yeast two-hybrid assays failed to detect an interaction between LUH-AD (full-length LUH fused to the GAL4 activation domain) and LUG-BD (full-length LUG fused to the GAL4 DNA-binding domain; Fig. 7A), nor could LUG homodimerize (Fig. 7B). Thus LUG and LUH likely act independently or in parallel to regulate common as well as unique target genes.

Previously, LUG was reported to interact directly with SEU via the N-terminal LUFS domain (Sridhar et al., 2004; Fig. 7, A and B). Because of the 80% sequence identity between LUG and LUH at the LUFS domain, we tested whether LUH could also interact with SEU. A strong interaction was detected between full-length LUH-AD and SEU(ND)-BD (Fig. 7B). SEU(ND) is a truncated SEU with its C-terminal domain (capable of self-activation) removed. Therefore, like LUG, LUH is likely able to form a corepressor complex with SEU.

DISCUSSION

LUG and *LUH* Exhibit Partially Redundant But Not Identical Functions

In this study, we investigated the function of *LUH*, the closest homolog of *LUG* in Arabidopsis. We discovered that these two genes play redundant roles during embryo development, revealing a previously unknown role of *LUG* during embryonic development. Second, we identified a relatively minor role of *LUH* compared with *LUG* in flower development, as the *luh-1* single mutation does not affect flower development but *luh-1/+* can enhance the floral phenotype of *lug*. Third, since overexpressing *LUH* could not rescue the weak *lug-16* mutation, the divergence in their coding sequences rather than their expression level or pattern likely contributes to their functional differences.

LUH and *SEU* Likely Act in the Same Pathway to Regulate Flower Development

We showed that both LUG and LUH interact physically with SEU in yeast, suggesting the possibility of forming both LUG/SEU and LUH/SEU corepressor complexes. Interestingly, *lug* and *luh* mutations exhibited drastically different genetic interactions with *seu*. Specifically, *lug; seu* double mutants exhibited a synergistic genetic interaction (Conner and Liu, 2000; Franks et al., 2002). In contrast, *seu-1* mutant flowers are indistinguishable from *seu-1; luh-1* double mutant flowers, suggesting that *seu-1* is completely epistatic to *luh-1* and implying that the function of *LUH* in flower development is entirely dependent on *SEU*. The yeast two-hybrid interactions detected between LUH and SEU suggest that LUH likely functions exclusively in a LUH/SEU complex.

LUG and *LUH* May Have Divergent Functions in Environmental Stress Responses

Recent genome-wide transcriptome studies comparing wild-type and *lug-3* mutant tissues revealed dramatic changes in the expression of genes involved in abiotic and biotic stress responses (Gonzalez et al.,

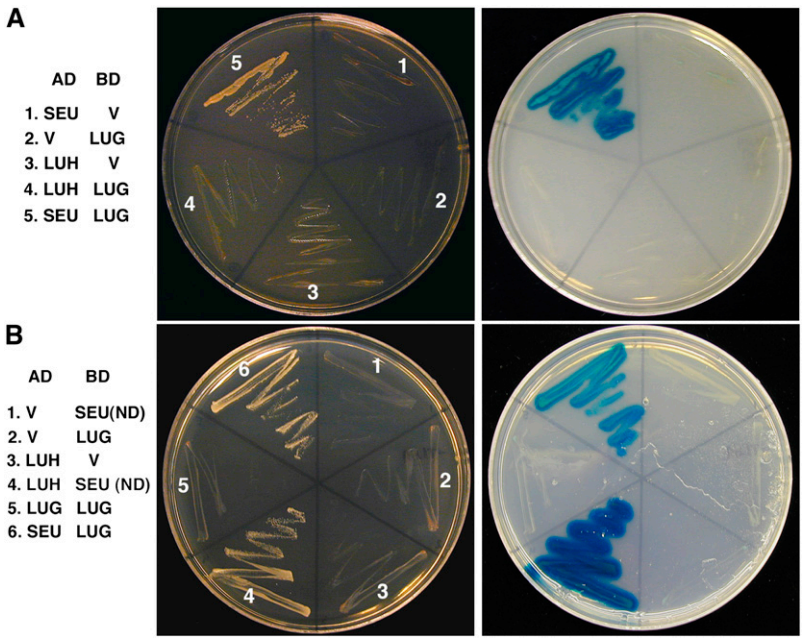

Figure 7. LUH interacts with SEU but not with LUG in yeast. A, Yeast two-hybrid assay showing a lack of interaction between LUH and LUG. Positive interaction is indicated by the activation of *HIS3* and *ADE2* reporter genes allowing colony growth on −Trp, −Leu, −His, and −Ade plates containing 3 mM 3-amino-1,2,4-triazole (left). The activity of a third reporter gene, *LacZ*, encoding β-galactosidase was tested by the X-Gal overlay assay (right); blue color indicates a positive interaction. B, Yeast two-hybrid assay showing a positive interaction between LUH and SEU(ND). V, Vector. [See online article for color version of this figure.]

2007). It is thus of particular interest to note the almost opposite expression trends between *LUG* and *LUH* under different biotic and abiotic challenges. This difference in gene expression patterns between *LUH* and *LUG* upon exposure to different environmental conditions (Fig. 6B) is in sharp contrast to the highly similar gene expression patterns between *LUH* and *LUG* in different tissues and developmental stages (Fig. 6A). This suggests that substantial differences may have occurred in the cis-regulatory elements of *LUH* and *LUG* involving responses to environmental signals.

Gene duplications are important evolutionary strategies in facilitating species adaptation, buffering deleterious mutations, subdividing their function, or evolving new functions (Lynch and Force, 2000; Lynch et al., 2001). Based on analyses of 2,022 recent duplicated gene pairs in Arabidopsis, duplicate genes with functions in developmental processes were found to be largely coregulated, while duplicate genes acting in abiotic or biotic stress responses were found to exhibit divergent expression profiles (Ha et al., 2007). This is consistent with our finding that *LUG* and *LUH* showed similar expression profiles during development but exhibited almost opposite expression trends when challenged with various environmental stresses. Our observation suggests that *LUG* and *LUH* may have substantially divergent functions when they act in stress response pathways.

A Proposed Model

Previous molecular and genetic characterizations of *lug* and *seu* mutants have been focused on flower development, which revealed important mechanistic insights into how *LUG* and *SEU* negatively regulate *AG* expression (Liu and Meyerowitz, 1995; Conner and Liu, 2000; Franks et al., 2002; Sridhar et al., 2004, 2006). SEU was shown to function as an adaptor protein bridging the interaction between the LUG corepressor and two MADS box transcription factors, AP1 and SEP3. AP1 and SEP3 recruit the SEU/LUG complex to *AG* by binding directly to the enhancer elements located in the second intron of *AG*. These previous studies laid important groundwork for our study and served as the basis for the model proposed below. Because of limited data on the function of *LUG*, *LUH*, and *SEU* in nonfloral tissues, the model (Fig. 8) is focused on the regulation of *AG* in flower development.

Previous synergistic genetic interactions between *lug* and *seu* in flowers (Franks et al., 2002) suggested that SEU serves as an adaptor for LUG as well as for other corepressors and that LUG may utilize SEU as well as other adaptor proteins. Thus, removing both SEU and LUG in *seu; lug* double mutants has a more severe effect. In contrast, the similar phenotype between *seu* single and *seu; luh-1* double mutants suggests that the function of LUH in flower development is dependent entirely on forming a complex with SEU. Therefore, we propose that LUG could form a corepressor complex with SEU as well as SEUSS-like (SLK) proteins (Franks et al., 2002) but that LUH could only pair with SEU. LUG/SEU and LUG/SLK complexes are either more prevalent or exhibit a higher repressor activity or both than LUH/SEU. As a result, LUG plays a more prominent role than LUH in the negative regulation of *AG*. This is illustrated in Figure 8, where in *luh* mutants, the LUG/SEU and LUG/SLK complexes are sufficient to cover the loss of LUH/SEU. In *seu* single or *seu; luh* double mutants, the LUG/SLK complex can still provide most if not all of the function. In *lug* mutants, LUH-SEU can also perform most

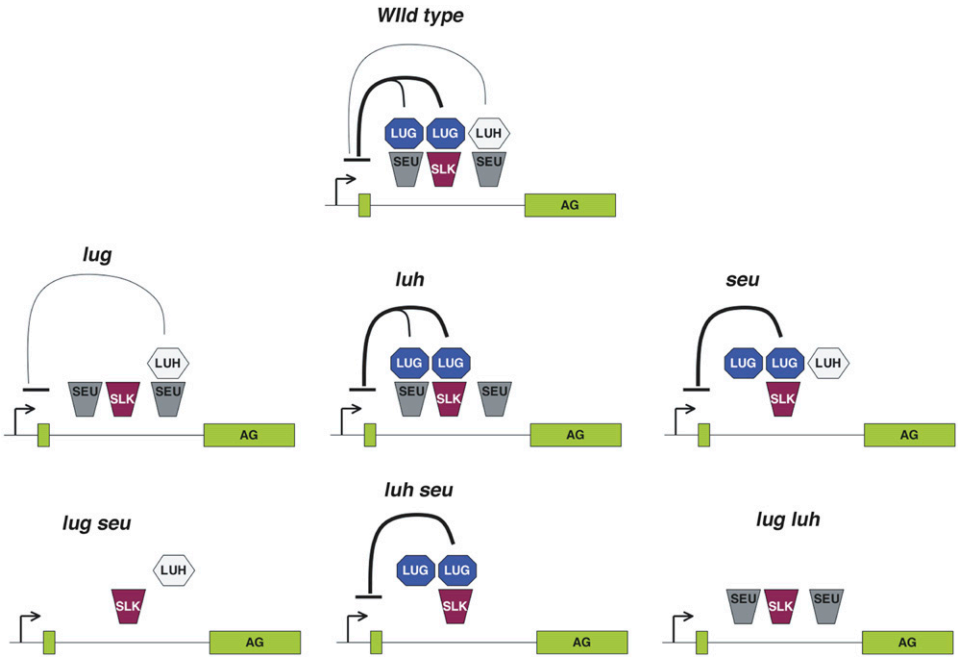

Figure 8. A model of the repression of *AG* by *LUG*, *LUH*, and *SEU* during flower development. The LUG/SEU, LUG/SLK, and LUH/SEU putative repressor complexes all act through the second intron of *AG*. The arrows indicate the transcription initiation from the *AG* promoter. Bars indicate repressor activity. Thick lines connecting to the bars indicate stronger repressor activity than thin lines. The specific repressor complexes are indicated in single and double mutant combinations. [See online article for color version of this figure.]

of the jobs. In *seu; lug*, or *luh; lug* double mutants, however, none of the LUH/SEU, LUG/SEU, or LUG/SLK complex is formed, leading to a much enhanced defect in the repression of AG and explaining the similar mutant floral phenotype between *seu-1; lug-3* and *luh-1/+; lug-3/lug-3* (Fig. 3; Franks et al., 2002).

CONCLUSION

Plant Gro/Tup1-type corepressors constitute an important class of regulatory molecules with roles in embryo shoot-root axis determination, stem cell pool maintenance, and floral homeotic gene regulation. Among the 13 Gro/Tup1-type corepressors in Arabidopsis, LUG and LUH are most similar to each other. We show that *LUH* and *LUG* exhibit both redundant and divergent functions in embryonic development, floral homeotic gene regulation, and plant biotic and abiotic stress responses. Gene duplication and functional diversification are important for species adaptation. Our study provides important insights into the complexity in the relationship between two highly homologous paralogous genes.

MATERIALS AND METHODS

Plant Growth and Mutant Identification

Arabidopsis (*Arabidopsis thaliana*) plants were grown on Sun Shine professional soil in controlled growth chambers at 20°C and 55% humidity under long-day (16 h of light) conditions. Seeds used in germination and root elongation assays were sterilized with 70% ethanol and 0.6% hypochlorite (bleach), plated on MS basal medium plates, incubated in the dark for 3 d at 4°C, and then grown for 5 d at 20°C under long days before transferring to another MS plate for root analyses.

lug-3, *lug-16*, and *seu-1* were generated in the Landsberg *erecta* background and were described previously (Conner and Liu, 2000; Franks et al., 2002). *luh-1* (*luh*_172H3; Arabidopsis Biological Resource Center stock no. CS91893) and *luh*-2 (*luh*_147A6; Arabidopsis Biological Resource Center stock no. CS91036) were generated by the Arabidopsis TILLING Project by ethyl methanesulfonate mutagenesis in the Columbia *erecta*-105 background (McCallum et al., 2000). *luh*-3 (SALK_107245) was generated by T-DNA insertion (Alonso et al., 2003).

Double Mutant Construction and Genotyping

To generate double mutants, *luh-1* pollen was used to pollinate *lug-16*, *lug-3*, or *seu-1*. F2 plants were analyzed by PCR-based genotyping methods. The *luh-1* dCAPS marker uses primers 5′-GCACCTGGAGGGTTTCCATTTGAGTG-3′ and 5′-CGCTTTACCTTGTTGTGCCTAAAATT-3′ in 35 cycles of PCR at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. PCR products (6 μL) were digested with *Bst*XI at 55°C and analyzed on 2.5% agarose gels. *luh-1* PCR products were resistant to *Bst*XI. *seu-1* dCAPS primers (Franks et al., 2002) amplified genomic DNA at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s for 35 cycles. The PCR products were digested with *Rsa*I. *seu-1* PCR products are resistant to the *Rsa*I digestion.

Since the dCAPS assay was not always reliable, an alternative fluorescence-based SNP assay (Amplifluor SNP Genotyping) was adopted for *luh-1* and *lug-16*. An individual leaf or single embryo was pressed onto FTA MicroCard (Whatman). A 0.2-mm-diameter disc was punctured out of the FTA MicroCard and served as a template for PCR following the manufacturer's protocol. Primers for *lug-16* (5′-GTTAAGTAGGAAGTTAAGCCC-3′ and 5′-GAGAACACCATTCAACTGTAC-3′) and *luh-1* (5′-GTTTGGGCTTTTATTCAGGTT-3′ and 5′-GCACTAGCATTAGACTGCCC-3′) were first used in a conventional PCR. Then, 25 ng of diluted PCR products served as templates for the Amplifluor SNP Genotyping system (assay development kit from Chemicon International, a subsidiary of Serologicals) using Platinum Taq DNA polymerase (Invitrogen). The Amplifluor AssayArchitect program (Chemicon International) was used for primer design; the allele-specific primer has a tail sequence complementary to either fluorescent FAM- or JOE-labeled primer. For the *lug-16* locus (tail sequence underlined), the wild-type-specific primer is 5′-GAAGGTGACCAAGTTCATGCTTCACCAGGTGCGTCAATAGCT-3′ and the *lug-16*-specific primer is 5′-GAAGGTCGGAGTCAACGGATTTCCACCAGGTGCGTCAATAGT-3′. Both allele-specific primers pair with the same reverse primer, 5′-CTGCAGTTGCTCTGTTTCCTAA-3′. All three primers were used in the same PCR genotyping procedure. For the *luh-1* locus (tails underlined), the wild-type-specific primer is 5′-GAAGGTCGGAGTCAACGGATTTGTCCCAAAACACAGACCAC-3′ and the *luh-1*-specific primer is 5′-GAAGGTGACCAAGTTCATGCTAATGTCCCAAAACACAGACCAT-3′. The reverse primer is 5′-GCACCTGGAGGGTTTCTTTTT-3′. PCR was run on a conventional PCR machine programmed as follows: (1) 96°C for 4 min; (2) 96°C for 12 s; (3) 57°C for 5 s; (4) 72°C for 10 s; (5) repeat steps 2 to 4 for 15 cycles; (6) 96°C for 12 s; (7) 55°C for 20 s; (8) 72°C for 40 s; (9) repeat steps 6 to 8 for 19 cycles; (10) 72°C for 3 s; and (11) hold at 20°C. Allelic discrimination was determined by reading FAM and JOE fluorophore signals using the Bio-Rad iQ5 PCR machine.

Microscopy and Photography

Floral, silique, and seedling photographs were captured with a Nikon SMZ1000 microscope equipped with a Nikon digital camera. The green and white seeds were dissected from siliques and fixed in Hoyer's solution for 15 min (Liu and Meinke, 1998) and then examined and photographed with a Nikon ECL1PSE E600W microscope with Nomarski optics and equipped with a DXM1200 digital still camera. Images were processed with Adobe Photoshop version 7.0.

Molecular Analyses of *LUH*

LUH (At2g32700) has 17 exons. 5′ RACE was performed to verify the *LUH* transcript using the Generacer kit (version F; Invitrogen) and total RNA from Arabidopsis flowers. The 5′ nested primer 5′-GGACACTGACATGGACTGAAGGAGTA-3′ was used, and the RACE products were cloned in pCRII TOPO (Invitrogen) and sequenced. LUH full-length cDNA (RAFL09-12-E08 [R12254]) was obtained from RIKEN Genomic Sciences Center and sequenced for confirmation.

To generate *35S::LUH*, the full-length *LUH* cDNA from RIKEN was amplified by PCR with primers 35SLUH-F (5′-ATTACCCGGGGATGGCTCAGAGTAATTGGGAAG-3′) and 35SLUH-R (5′-TCCCCCGGGCTACTTCCAAATCTTTACGGA-3′) containing engineered *Xma*I sites with the high-fidelity Taq polymerase (Roche). The PCR product was cloned in the pBI121 vector at the *Xma*I site and verified by sequencing. Plasmids were transformed into *Agrobacterium tumefaciens* GV3101 through electroporation. *luh-1* and *lug-16* plants were transformed by the floral dip method (Clough, 2005). Kanamycin-resistant T1 seedlings were identified on MS plates containing 50 μM kanamycin and transferred to soil.

For RT-PCR, RT was performed with oligo(dT) and SuperScript II reverse transcriptase enzyme (Invitrogen). All RT-PCR procedures were carried out for 25 cycles and were repeated at least twice. Primers used were *LUH* (5′-TGGCTCAGAGTAATTGGGAAG-3′ and 5′-CCAGGCTTTGATTGCAGA-3′) and *ACT2* (5′-GTTGGGATGAACCAGAAGGA-3′ and 5′-CTTACAATTTCCCGCTCTTC-3′). The primers were designed to span introns to avoid amplification from contaminated genomic DNA. *ACT2* was used as a loading control. The RT-PCR procedures were quantified using ImageQuant 1.1 (National Institutes of Health) software, based on the intensity of the ethidium bromide staining.

Yeast Two-Hybrid Assay

Full-length *LUH* cDNA was amplified by PCR using high-fidelity Taq polymerases (Roche) and the RIKEN (RAFL09) cDNA as a template with engineered primers. PCR products were cloned into the pCRII TOPO vector (Invitrogen). The clone was sequenced to verify amplification accuracy. The primers LUH-BD-f and LUH-AD-f, which are the same (5′-ATTACCCGGGGATGGCTCAGAGTAATTGGGAAG-3′), LUH-BD-r (5′-ACGCGTCGACATCTACTTCCAAATCTTTACGGA-3′), and LUH-AD-r (5′-ATTCTCGAGCTACTTCCAAATCTTTACGGA-3′) contain *Sal*I and *Xma*I sites for the BD fusion and *Xho*I and *Xma*I sites for the AD fusion.

The *LUH* fragments were excised from corresponding pCRII TOPO vectors and inserted into pGBKT7 and pGADT7 (Clontech), respectively, at corresponding enzyme sites. The yeast host (PJ69-4A), genotype MAT**a** trp1-901 leu2-3,112 ura3-52 his3-200 gal4delta gal80delta GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ (James et al., 1996), was used for transformation as described previously (Sridhar et al., 2004).

For the X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) overlay assay, 0.125 g of agarose was dissolved in 25 mL of sterilized Z-buffer (60 mM $Na_2HPO_4 \cdot 2H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, and 1 mM $MgSO_4 \cdot 7H_2O$, pH 7.0) by heating in a microwave oven. After cooling to 50°C, 0.5 mL of 10% SDS and X-Gal dissolved in *N*,*N*-dimethylformamide (final concentration, 2 mg/mL) were added. The molten agarose solution was poured over one to two plates containing yeast colonies. After 30 min of incubation at 37°C, the plates were photographed.

Supplemental Data

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

Supplemental Figure S1. Sequence of LUH cDNA and deduced amino acid sequence.

Supplemental Table S1. Percentage of abnormal seeds (white seeds) in the wild type and mutants.

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