**SEUSS**, a member of a novel family of plant regulatory proteins, represses floral homeotic gene expression with **LEUNIG**

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**SUMMARY**

Proper regulation of homeotic gene expression is critical for pattern formation during both animal and plant development. A negative regulatory mechanism ensures that the floral homeotic gene **AGAMOUS** is only expressed in the center of an **Arabidopsis** floral meristem to specify stamen and carpel identity and to repress further proliferation of the floral meristem. We report the genetic identification and characterization of a novel gene, **SEUSS**, that is required in the negative regulation of **AGAMOUS**. Mutations in **SEUSS** cause ectopic and precocious expression of **AGAMOUS** mRNA, leading to partial homeotic transformation of floral organs in the outer two whorls. The effects of **seuss** mutations are most striking when combined with mutations in **LEUNIG**, a previously identified repressor of **AGAMOUS**. More complete homeotic transformation of floral organs and a greater extent of organ loss in all floral whorls were observed in the **seuss leunig** double mutants. By in situ hybridization and double and triple mutant analyses, we showed that this enhanced defect was caused by an enhanced ectopic and precocious expression of **AGAMOUS**. Using a map-based approach, we isolated the **SEUSS** gene and showed that it encodes a novel protein with at least two glutamine-rich domains and a highly conserved domain that shares sequence identity with the dimerization domain of the LIM-domain-binding transcription co-regulators in animals. Based on these molecular and genetic analyses, we propose that **SEUSS** encodes a regulator of **AGAMOUS** and functions together with **LEUNIG**.

Key words: **LEUNIG**, **AGAMOUS**, **APETALA2**, Co-repressor, Flower development, LIM domain binding protein, **Arabidopsis thaliana**

**INTRODUCTION**

Pattern formation and organ morphogenesis represents one of the most challenging and important questions in developmental biology. The ABC model of flower development elegantly explains how the identity of the four types of floral organs is specified (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). An **Arabidopsis** flower consists of four types of floral organs arranged in four concentric whorls. Four sepals develop in the outermost whorl (whorl 1), four petals develop in whorl 2, six stamens arise in whorl 3, and two carpels fuse with each other to form a gynoecium in whorl 4. The A, B and C classes of floral homeotic genes (also termed ‘organ identity genes’) function in specific and adjacent whorls to specify floral organ type. The mRNAs of most A, B and C genes are expressed only in the floral whorls where their activities are required (Drews et al., 1991; Jack et al., 1992; Mandel et al., 1992; Goto and Meyerowitz, 1994). Hence, proper transcriptional regulation of the A, B and C genes is crucial to the proper specification of floral organ type. Revealing the regulatory mechanism underlyling floral homeotic gene expression, thus, poses the next major challenge in the field.

The regulation of the C class floral homeotic gene **AGAMOUS** (**AG**) is the most extensively studied. In **ag** loss-of-function mutants, stamens are replaced by petals, and carpels are replaced by a new flower. The generation of flowers within a flower reveals a second role of **AG** in maintaining the determinacy of the floral meristem (Bowman et al., 1989; Bowman et al., 1991; Mizukami and Ma, 1997). **AG** encodes a DNA-binding transcription factor of the MADS box family (YanoFSky et al., 1990; Huang et al., 1993). In wild-type, **AG** mRNA is only turned on at stage 3, when the sepal primordia just arise from the floral meristem (Smyth et al., 1990), and is only detected in the inner two whorls of a flower (YanoFSky et al., 1990; Drews et al., 1991). Its precise regulation requires the activity of both positive regulators **LEAFY** (**LFY**), **APETALA1** (**AP1**) and **WUSCHEL** (**WUS**) and negative regulators such as **LEU NicolasL** (**LUG**) and **APETALA2** (**AP2**) (Bowman et al., 1991; Drews et al., 1991; Weigel et al., 1992; Weigel and Meyerowitz, 1993; Liu and Meyerowitz, 1995; Lennard et al., 2001; Lohmann et al., 2001). **LFY** and **WUS** were shown to bind directly to the second intron of **AG** and activate **AG** expression (Busch et al., 1999; Lohmann et al., 2001). However, the mechanism of negative regulation is less well understood.
**Table 1. CAPS and dCAPS markers used in this study**

<table>
<thead>
<tr>
<th>Marker name</th>
<th>Oligonucleotide sequence</th>
<th>Restriction enzyme</th>
<th>Restriction fragments for Ler (bp)</th>
<th>Restriction fragments for Col (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2J6i (CAPS)</td>
<td>Forward: 5’CACATTGGATGATCGAAGA3’ Reverse: 5’TGACTTCCTCCTGGGCTTCT3’</td>
<td>AclI</td>
<td>239 117 33</td>
<td>272 117</td>
</tr>
<tr>
<td>F28H19i (CAPS)</td>
<td>Forward: 5’TTCCTGGAGAAGAAGTTGTGT3’ Reverse: 5’AACTCCATTGAGCACAAGA3’</td>
<td>MseI</td>
<td>395</td>
<td>320 75</td>
</tr>
<tr>
<td>F9L16Sp6 (CAPS)</td>
<td>Forward: 5’CTAGTGATGATCCTGGAGA3’ Reverse: 5’GAGTGGGTGCTTCTGCTTC3’</td>
<td>DraI</td>
<td>784 382</td>
<td>692 382</td>
</tr>
<tr>
<td>F27F5ii (CAPS)</td>
<td>Forward: 5’TCTGGACGTAAGACACAAA3’ Reverse: 5’TCCTTGTCACAAACCTATC3’</td>
<td>MseI</td>
<td>405 200 143 75</td>
<td>553 143 75 54</td>
</tr>
</tbody>
</table>

**Materials and Methods**

**Genetic analysis**

Both seu-1 and seu-2 were induced by EMS in the Landsberg erecta (Ler) ecotype, seu-1 was isolated in a screen for genetic enhancers of unusual floral organs (Levin et al., 1998), seu-2 was isolated in a screen for enhancers of crabs claw (Eshed et al., 1999). Both seu-1 and seu-2 were back-crossed to wild type (Ler) three times before further genetic and phenotypic analyses.

LUG, AP2 and AG all reside on chromosome 4 in the following order: AP2 (16 CM) LUG (10 CM) AG. SEU resides on chromosome 1 (see below). To generate seu lug and seu ap2 double mutants, seu-1 homozygous flowers were fertilized with pollen from lug-1, lug-2, lug-3, lug-8, ap2-1 or ap2-2, respectively. Seeds were collected from F3 seu individuals (selected based on plant height and floral defects) and the respective double mutants were observed in 1/4 of the F3 plants. To generate the seu-1 lug-1 ag-1 triple mutants, ag-1/+ plants were fertilized with lug-1 pollen. F2 lug-1 plants were crossed to seu-1/lug-1, ap2-1/lug-2 double mutants to generate seu-1+/+; lug-1 ag-1/lug-1 ap2-1 + +. Approximately 10% of the F2 families from this second cross segregated lug-1 ag-1 double mutants in 3/16 of the progeny and the seu-1 lug-1 ag-1 triple mutant in 1/16 of the progeny. The genotype of the ag-1 lug-1 seu-1 triple mutant was confirmed by Co-Dominant Amplified Polymorphic Sequences (CAPS)- or dCAPS-based markers (Konieczny and Ausubel, 1993; Neff et al., 1998) developed for lug-1 and seu-1 (Table 1). To generate seu-1/lug-1, lug-1 +/+ ap2-1 plants, lug-1 individuals were crossed to seu-1 ap2-1 double mutants. F2 families segregated seu-1/lug-1, lug-1 +/+ ap2-1 individuals with an enhanced seu phenotype in 1/8 of the progeny. The genotypes of these plants were confirmed by CAPS or dCAPS markers developed for seu-1, ap2-1 and lug-1 (Table 1).

**Microscopic analyses**

Scanning electron microscopy (SEM) samples were collected, fixed
Positional cloning of SEU

A mapping population was generated by crossing seu-1/seu-1 of the Ler ecotype with wild-type Columbia (Col) ecotype. Genomic DNA was isolated from 305 of the F2 seu-1 plants and assayed by PCR-based markers. Linkage of SEU to the chromosome 1 markers GAPB and F16N3 led to the physical map (Fig. 5A). Finer mapping subsequently placed SEU 0.16 cM north of the marker F9L16Sp6 (Fig. 5A; Table 1). This placed SEU on the BAC clone F28H19 (AC006423). Sequencing and annotation by the Arabidopsis Genome Initiative predicts 13 open reading frames (ORFs) on F28H19. Among these ORFs is a glutamine-rich protein (F28H19.10). Fragments spanning the F28H19.10 ORF were amplified by PCR from seu-1 and seu-2 plants, respectively, and then directly sequenced. The mutational changes in the seu-1 and seu-2 alleles were confirmed by repeating the amplification and sequencing analysis.

PCR analyses using CAPS and dCAPS markers

dCAPS markers (Neff et al., 1998) were designed for both seu-1 and seu-2 alleles based on the mutations in seu-1 and seu-2 respectively (Fig. 5A), dCAPS marker for ap2-1 was designed based on sequences published by Jofuku et al. (Jofuku et al., 1994). The CAPS marker for lag-1 was based on Conner and Liu (Conner and Liu, 2000). These dCAPS or CAPS markers correctly distinguish wild-type from the mutant plants. The primer sequence for each marker is listed in Table 1. PCR amplification was performed under standard conditions.

Molecular analyses of SEU

A 5’ rapid amplification of cDNA ends (5’RACE) was carried out using the 5’ RACE kit-version 2.0 (Gibco/BRL). Three nested primers from the 5’ gene-specific region of SEU were used: oligo 923, 5’AAACCACTAAACCCGAGCCT3’; oligo 265, 5’GGGTCAGAGTCAGCACCACT3’; oligo 178, 5’ATTATTGAGGACATTCAGCAAGC3’. 5’RACE products were cloned into pCRII-TOPO vector (Invitrogen) and sequenced. Blast searches identified six SEU EST clones. Five clones (AV521646, AV522370, AV531945, AV546257, AV535478) were provided by the Kazusa DNA Research Institute and one clone (AI997332) was purchased from Genome Systems Inc./Incyte Pharmaceuticals Inc. AV546257 is a full-length cDNA clone.

In situ experiments were performed as previously described (Liu et al., 2000). For northern analyses, total RNA was isolated using the Tri-reagent RNA isolation system (Sigma) from leaves and inflorescences containing flowers at all stages. mRNA was subsequently isolated from the total RNA using the polyATrack mRNA isolation system III (Promega). 2.5 µg mRNA was fractionated on 1% denaturing formamide-agarose gels, blotted, hybridized and washed according to the method of Ausubel et al. (Ausubel et al., 1991). A 874 bp KpnI fragment corresponding to the 3’ end of SEU (2685-3559 bp) was used as a probe.

RESULTS

seu mutants exhibit defects in floral organ identity and organ number

EMS-induced seu-1 and seu-2 mutants (Materials and Methods) initially appeared similar in the severity of their phenotypes. Further characterization showed that seu-2 was slightly stronger than seu-1. For both alleles, late-arising flowers exhibited more severe phenotypes than early-arising flowers. In the late-arising flowers, the organ number in whorls 2 and 3 is reduced (Fig. 1B,C; Table 2). On average, only 3 organs are found in whorl 2, and 5 organs in whorl 3. 7% of

![Fig. 1. Phenotypes of seu mutant plants. A-C,I are photographs; D-H and J-K are scanning electron micrographs. (A) A wild-type flower. (B) A seu-1 mutant flower with narrow petals (arrow) and narrow sepals (arrowhead). (C) A seu-2 mutant flower exhibiting a whorl 1 stamen/carpel mosaic organ (arrowhead) and a whorl 2 petal that is reduced in size and is staminoid (arrow). The tip of the gynoecium is unfused, exposing the ovules (o). (D) Abaxial surface of a seu-1 whorl 1 organ. Both petal (pe) and sepal (se) cells are present. (E) An enlargement from the boxed area in D. Both round-shaped petal cells (arrow) and rectangular-shaped sepal cells (arrowhead) are present. (F) Wild-type petal cells. (G) Wild-type sepal cells. (H) A seu-2 mutant flower. Note the long tubular organs (arrows) in whorl 2. These tubular organs are likely petal in identity because of the petal blade at the tip of the tube. (I) Wild-type and mutant plants. All plants are Ler ecotype. The ruler is 15 cm long. The seu-1 plant is about half the height of the wild-type one; seu-1 ap2-1 is similar in height to seu-1; seu-1 lug-8 double mutant is less than half the height of seu-1 and is bushy. Inset box is an enlarged picture of a seu-1 lug-8 plant. (J) A mature wild-type ovule. (K) A seu-1 ovule with an abnormal protrusion at the micropylar end (arrowhead). Scale bars in D, E, G, J, K are 100 µm; bar in F is 50 µm; bar in H is 1 mm.](image-url)
whorl 1 organs display partial homeotic transformation and possess sepal/petal or sepal/carpel mosaics (Fig. 1C-G; Table 2). Whorl 2 organs are most often narrow petals, but stamenoid petals were occasionally observed (Fig. 1C). Alternatively, petals can be replaced by filamentous or tubular structures (Fig. 1H). Whorl 3 stamens are typically reduced slightly in size. The whorl 4 gynoecium is often slightly split at the top (Fig. 1C, Fig. 2I). Sometimes, horn-like protrusions are seen at the gynoecium apex (data not shown).

In addition to defects in floral organ identity and organ number, seu plants exhibit other defects including narrow floral organs (Fig. 1B-C, Fig. 2I), narrow leaves (data not shown), reduced plant height (Fig. 1I) and increased lateral branching (Fig. 1I). Furthermore, the number of seeds per silique is reduced. On average, seu-1 produces 18.1±6.2 (n=15) seeds per silique while wild-type (Ler) produces 62±7.4 (n=11) seeds per silique. Occasionally, seu-1 ovules develop abnormally with a protrusion from the micropylar end (Fig. 1I,K). The seu phenotype indicates that SEU plays diverse roles during plant development.

**Seu genetically enhances lug**

With the exception of the reduced plant height, the floral, ovule, and vegetative defects of seu mutants are similar to, but weaker than, those of lug. To understand the relationship between seu and lug, we generated and characterized seu lug double mutants. Seu lug double mutant flowers display a dramatically enhanced phenotype characterized by a reduction in flower size and floral organ number and an enhanced carpelloidy of whorl 1 organs (Fig. 2B-D; Table 2). Most often, only two whorl 1 organs are formed. These whorl 1 organs are often carpelloid as evidenced by their epidermal cell morphology, the formation of horns (a character of lug carpels), and the expression of AG (see later). Whorl 2 organs are completely absent. In whorl 3, one stamen is occasionally formed, averaging 0.4 per flower. Whorl 4 carpels develop into a small stub or mound of tissues. Occasionally, structures derived from carpel marginal meristems (i.e. ovule, stigma, style, and septum) are not observed on whorl 1 carpelloidal organs, nor on whorl 4 mounds (Fig. 2B,C). An exception to this is the seu-1 lug-8 double mutant (lug-8 is a weak allele).
where some ovule primordia were occasionally observed in either whorl 1 organs or whorl 4 mounds (Fig. 2D). Vegetative defects were also enhanced in the seu lug double mutants (Fig. 1I). Although lug single mutations have no effect on plant height, seu-1 lug-1 double mutants are only 12% of wild-type height (2.7±0.9 cm; n=20), much shorter than seu-1 (11.4±1.6 cm; n=10). In summary, the seu lug double mutant flowers exhibit increased carpelloidy in whorl 1, enhanced organ loss in whorls 1-3, a reduction of whorl 4 gynoecium, and a loss of carpel marginal tissues. An overall reduction of flower size and plant height was also observed.

**seu genetically enhances ap2**

Since AP2 plays a major role in AG repression and ap2 interacts with lug synergistically (Bowman et al., 1991; Liu and Meyerowitz, 1995), we sought to determine the relationship between ap2 and seu. Both weak ap2-1 and strong ap2-2 alleles were used for the analysis. The weak ap2-1 flower develops leaf-like whorl 1 organs and staminoid whorl 2 petals (Fig. 2E) (Bowman et al., 1989; Bowman et al., 1991). In seu-1 ap2-1 double mutants, first whorl organs are converted to carpelloid structures as evidenced by the presence of stigmatic tissue and ovule primordia and the absence of leaf-like trichomes (Fig. 2F). In the strong ap2-2 flowers, whorl 1 organs are carpelloid, whorl 2 and 3 organs are absent, and in whorl 4 a relatively normal gynoecium is formed (Fig. 2G) (Bowman et al., 1989; Bowman et al., 1991). Similar to ap2-2, seu-1 ap2-2 double mutant whorl 1 organs are carpelloid (Fig. 2H). However, the seu-1 ap2-2 whorl 1 carpels have less stigmatic tissue and fewer ovules than ap2-2. The most obvious difference between ap2-2 single mutant and seu-1 ap2-2 double mutant is in whorl 4 where only a small mound of tissue develops (Fig. 2H). Hence, seu enhances the defects of weak ap2-1 in homeotic transformation and organ loss and enhances

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**Table 2. Effects of seu, lug, ap2 and ag mutants on organ number and organ identity**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of organs in whorl 1</th>
<th>Number of organs in whorl 2</th>
<th>Number of organs in whorl 3</th>
<th>Number of organs in whorl 4</th>
<th>% of whorl 1 organs displaying homeotic transformation</th>
<th>Number of flowers examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>4.0±0.0</td>
<td>4.0±0.0</td>
<td>5.8±0.39</td>
<td>2.0±0.0</td>
<td>0%</td>
<td>17</td>
</tr>
<tr>
<td>seu-1/seu-1</td>
<td>3.8±0.4</td>
<td>3.0±1.0</td>
<td>4.8±0.90</td>
<td>2.0±0.18</td>
<td>7.4%*</td>
<td>86</td>
</tr>
<tr>
<td>seu-1/seu-1; ap2-2/+</td>
<td>4.0±0.0</td>
<td>2.2±0.77</td>
<td>4.4±0.83</td>
<td>1.5±0.52</td>
<td>25%</td>
<td>15</td>
</tr>
<tr>
<td>seu-1/seu-1; lug-1/ap2-1</td>
<td>3.7±0.7</td>
<td>1.6±1.1</td>
<td>3.2±1.0</td>
<td>1.9±0.47</td>
<td>38%</td>
<td>14</td>
</tr>
<tr>
<td>lug-1/lug-1</td>
<td>4.0±0.0</td>
<td>0.7±0.72</td>
<td>2.7±0.72</td>
<td>1.7±0.49</td>
<td>40%</td>
<td>15</td>
</tr>
<tr>
<td>seu-1/seu-1; lug-1lug-1</td>
<td>3.2±0.68</td>
<td>0.0±0.0</td>
<td>0.4±0.91</td>
<td>0.0±0.0</td>
<td>88%</td>
<td>15</td>
</tr>
<tr>
<td>seu-1/seu-1; lug-8/lgug-8</td>
<td>2.9±0.47</td>
<td>0.4±0.94</td>
<td>1.2±0.97</td>
<td>0.4±0.65</td>
<td>ND</td>
<td>14</td>
</tr>
<tr>
<td>ag-1/lug-1</td>
<td>4.0±0.0</td>
<td>4.0±0.0</td>
<td>6.0±0.0</td>
<td>43±3.5**</td>
<td>0%</td>
<td>8</td>
</tr>
<tr>
<td>lug-1 ag-1/lug-1 ag-1</td>
<td>4.0±0.0</td>
<td>3.9±0.33</td>
<td>5.6±0.88</td>
<td>41±4.2**</td>
<td>0%</td>
<td>9</td>
</tr>
<tr>
<td>seu-1/seu-1; ag-1/lug-1</td>
<td>3.6±0.79</td>
<td>4.1±0.38</td>
<td>5.9±0.38</td>
<td>ND</td>
<td>0%</td>
<td>7</td>
</tr>
<tr>
<td>seu-1/seu-1; lug-1 ag-1/lug-1 ag-1</td>
<td>3.9±0.27</td>
<td>3.8±0.58</td>
<td>4.4±1.0</td>
<td>16.9±5.7**</td>
<td>9%</td>
<td>14</td>
</tr>
</tbody>
</table>

*Mostly petal/sepal mosaics.
† Mostly stamen/sepal or carpel/sepal mosaics.
‡ Similar to †, also see Liu and Meyerowitz (1995).
§ Mostly sepal to carpel transformations.
¶ Only petal/sepal mosaics.
**Number of organs formed interior to whorl 3.
ND, not determined; values are mean ± s.d.

*Fig. 3. Ectopic and precocious expression of AG in seu single and seu lug double mutants. 8 µm longitudinal sections of Arabidopsis inflorescences were hybridized with an AG antisense probe. Numbers indicate stages of floral development [based on Smyth et al. (Smyth et al., 1990)]. (A) A wild-type inflorescence. AG mRNA is detected in the center of the stage 5 flower and is not detected in sepal primordium (arrowhead). AG mRNA is not detected in stage 2 floral meristems. (B) A seu-1 inflorescence. AG mRNA is detected in the stage 2 floral meristem and in the sepal primordia of stage 3 floral meristem (arrowhead). (C) A seu-1 lug-8 double mutant inflorescence. AG mRNA is detected as early as the stage 1 and stage 2 floral meristems. Expression of AG mRNA is also detected in groups of cells (marked with a *) in the inflorescence meristem that might represent pre-stage 1 cells. AG mRNA is detected in the sepals of all flowers (arrowheads). Note the severely reduced whorl 4 (white arrows). The pink color in the stem is residual Eosin stain and does not reflect hybridization signal.*
the defects of strong ap2-2 primarily in the whorl 4 gynoecial development.

In the homozygous seu mutant background, the strong ap2-2 allele behaves as a dominant enhancer of seu. While seu-1/seu-1 plants display homeotic transformations in only 7.4% of whorl 1 organs, seu-1/seu-1; ap2-2/+ plants display homeotic transformations in 25% of whorl 1 organs (Table 2) with a greater degree of homeotic transformation (Fig. 2L,J). Furthermore, the lug-1 allele behaves as a dominant enhancer in the seu-1/seu-1; ap2-1/+ background. Carpelloid and staminoid transformations are observed in 38% of whorl 1 organs in seu-1/seu-1; lug-1 +/- ap2-1/+ flowers (Table 2; Fig. 2, compare K,L with J). In summary, the degree of mutant severity with respect to homeotic transformation can be ordered as follows: seu-1/seu-1 < seu-1/seu-1; ap2-2/+ < seu-1/seu-1; lug-1 +/- ap2-1/+ < ap2-2/ap2-2 (<; less severe than). Therefore, seu, lug and ap2 exhibit both synergistic and dominant genetic interactions.

**AG is ectopically expressed in seu single and seu lug double mutants**

To test if the carpelloid and staminoid homeotic transformation of whorl 1 organs and the reduction of organ number observed in the seu single and seu lug double mutant flowers are primarily caused by the ectopic expression of AG, we examined AG mRNA expression by in situ hybridization. In wild-type flowers, AG mRNA is first detected at stage 3 in the center of a floral meristem (Fig. 3A) (Drews et al., 1991). In contrast, AG mRNA was sometimes detected in all four whorls in stage 3 seu flowers (Fig. 3B). Additionally, AG mRNA was sometimes detected in stage 2 seu-1 floral primordia (Fig. 3B). Thus, seu causes both ectopic and precocious AG mRNA expression. In seu-1 lug-8 double mutant flowers, the ectopic AG expression was enhanced as shown both by a greater extent of ectopic AG expression in whorl 1 organs and by a higher percentage of whorl 1 organs that express AG (Fig. 3C). Most strikingly, precocious AG expression was detected in floral meristems as early as stage 1 or even in groups of cells that are about to form the stage 1 floral meristem (ie. pre-stage 1 cells) (Fig. 3C). This stage 1/pre-stage 1 expression of AG was never observed in lug or seu single mutants.

**Removing ectopic AG activity restores proper organ identity and organ number but not organ shape or plant height**

The above studies showed that the extent and severity of homeotic transformation and organ loss correlated with the extent of ectopic/precocious AG expression in seu and seu lug mutant flowers. By constructing seu ag double and seu lug ag triple mutants, we sought to determine if removing AG activity in the seu and seu lug background can restore proper organ identity, organ shape or organ number. We found that the organ identity and organ number of the seu-1 ag-1 flowers are similar to those of ag-1 flowers. Four sepals develop in whorl 1, and four petals develop in whorl 2 (Fig. 4A,B; Table 2). However, the petals and sepals of seu-1 ag-1 flowers are narrower than those of ag-1 or wild-type (Fig. 4B) and are similar to seu-1 flowers. Furthermore, plant height is similar in seu-1 ag-1 and seu-1 plants. Therefore, removing ectopic/precocious AG in seu background can restore defects in floral organ identity and organ number but not in organ shape or plant height.

In seu-1 lug-1 ag-1 triple mutant flowers, floral organ type and organ number in whorls 1-3 are similar to those in ag-1 flowers (Fig. 4C; Table 2). Although whorl 1 organs of the triple mutant are narrow and canoed-shaped, their epidermal cell morphology is characteristic of sepals (Fig. 4C). Occasionally (9%), some of these whorl 1 organs are sepal/petaloid mosaics but they are never carpelloid (Table 2). This is in contrast to the high percentage (88%) of whorl 1 carpelloid organs found in seu-1 lug-1 ag-1 double mutants (Table 2; Fig. 2B-D). The whorl 2 and 3 organs of seu-1 lug-1 ag-1 flowers are sometimes blade-like (Fig. 4C,E) with epidermal cell morphology characteristic of petals (Fig. 4D). Alternatively, the whorl 2-3 organs sometimes look like clubs (Fig. 4C,F) with epidermal cell morphology characteristic of the base of petals (data not shown). On average, 3.8 whorl 2 and 4.4 whorl 3 organs were observed in the seu-1 lug-1 ag-1 flowers as compared to zero whorl 2 and 0.4 whorl 3 organs in the seu-1 lug-1 double mutants (Table 2). Finally, the seu lug ag triple mutant (2.9 cm±0.8; n=13) is similar in height to the seu-1 lug-1 double mutant (2.7±0.9 cm; n=20). Thus, removing ectopic/precocious AG activity from seu lug double mutants restores correct floral organ identity and organ number in whorls 1-3 but does not restore normal floral organ shape or plant height.
Fig. 5. Molecular cloning of the SEUSS gene. (A) A physical map of the SEU region on Arabidopsis chromosome I. Percentage recombination for five CAPS markers is shown. n indicates the number of meiotic products examined at the given marker. BAC clones are shown as open boxes. The SEU region maps 0.16 cM north of the CAPS marker F9L16Sp6. (B) Nucleotide sequence and the predicted amino acid sequence of SEU. Numbers on the right indicate the amino acid residue. The boxed area encodes a bi-partite nuclear localization signal. The underlined sequence is the putative dimerization domain with similarity to the Ldb proteins. The filled triangles indicate seu-1 and seu-2 mutations. The seu-1 allele is caused by a C to T transition, resulting in a 'TAA' stop codon at amino acid 501. The seu-2 allele is caused by a single base-pair deletion of the 'G' base indicated. The full length cDNA sequence (3555 bp) including 5' and 3' UTR has been deposited with GenBank (AF378782).
transcriptional regulators including carboxypeptidase, and one glutamine-rich (Q-rich) protein putative acyl-acyl carrier protein desaturase, one serine are: three hypothetical proteins, one unknown protein, one of the marker F9L16Sp6. Six of these appeared to encode indicated the presence of 13 ORFs on F28H19 that were north of the marker GAPB. Finer mapping using additional Materials and Methods.

We isolated the results from an additive effect of the floral indeterminancy floral organ number in whorl 4 of the triple mutant likely only 16.9±5.7 organs interior to whorl 3 (Table 2). The reduced pattern, generating an average of 43±3.5 organs interior to whorl 4 ag-1 flowers, the whorl 4 gynoecium is replaced by an indeterminate flower that repeats the (sepal-petal-petal) n pattern, generating an average of 43±3.5 organs interior to whorl 3. In contrast, the seu-1 lug-1ag-1 triple mutant averaged only 16.9±5.7 organs interior to whorl 3 (Table 2). The reduced floral organ number in whorl 4 of the triple mutant likely results from an additive effect of the floral indeterminancy caused by ag-1 and the reduced whorl 4 primordium caused by the seu-1 lug-1 (Fig. 2B-D; Fig. 3C).

The SEU gene encodes a glutamine-rich protein with a putative dimerization domain

We isolated the SEU gene by positional cloning (Fig. 5; Materials and Methods). SEU was first mapped to the centromeric region of chromosome 1, approximately 2.4 cM south of the marker GAPB. Finer mapping using additional CAPS markers (Fig. 5A; Table 1) indicated that SEU resides 0.16 cM north of the CAPS marker F9L16Sp6. Our recombination data from this region of chromosome 1 indicate that 0.16 cM represents approximately 40 kb, thus SEU likely resides on BAC clone F28H19. Sequencing and annotation of F28H19 (AC006423) by the Arabidopsis Genome Initiative indicated the presence of 13 ORFs on F28H19 that were north of the marker F9L16Sp6. Six of these appeared to encode portions of transposable elements. The seven remaining ORFs are: three hypothetical proteins, one unknown protein, one putative acyl-acyl carrier protein desaturase, one serine carboxypeptidase, and one glutamine-rich (Q-rich) protein (F28H19.10). Because Q-rich sequences are found in many transcriptional regulators including LUG, F28H19.10 ORF was a likely candidate for SEU. Sequence analysis of the F28H19.10 ORF in seu-1 and seu-2 genomic DNA identified mutations in both alleles. In the seu-1 allele, a C to T transition results in the change of a glutamine codon to a stop codon at amino acid 501 (Fig. 5B). In the seu-2 allele, a single base pair deletion in codon 343 leads to a frame shift (adding 54 novel amino acid residues) and a subsequent stop codon (Fig. 5B). The nature of the mutational changes found in seu-1 and seu-2 strongly supports that F28H19.10 encodes the SEU gene.

Using the F28H19.10 ORF sequence as a query in a TBlastN search, six SEU Arabidopsis EST clones were identified. Based on sequence analysis of these EST clones and 5′ RACE, full length SEU cDNA is represented by two transcripts of 3555 bp and 3406 bp respectively. The shorter transcript initiates 149 bp downstream from the longer transcript. Northern analyses showed that the transcript levels in seu-1 and seu-2 mutants are reduced to 59% and 78% wild-type level respectively (Fig. 6A). This reduced mRNA level likely reflects a reduced mRNA stability in the two seu mutants, both of which are predicted to produce truncated SEU protein. Consistent with diverse developmental roles played by SEU, SEU mRNA is expressed in all tissues examined including flowers and leaves (Fig. 6B) as well as seedlings (data not shown).

SEU encodes a Q-rich (15% Q overall) protein of 877 amino acid (Fig. 5B). Two major Q-rich regions reside in residues 179-289 (42% Q) and 582-632 (61%Q), respectively (Fig. 5B, Fig. 7A). Within the second Q-rich region there is a stretch of 24 Q residues. A putative bipartite nuclear localization signal (Robbins et al., 1991) found between amino acids 330 and 344 (Fig. 5B) suggests that SEU likely resides in the nucleus. In addition, a 243 amino acid central domain (residues 321-563) is highly conserved. Between 21% and 81% amino acid sequence identity was found within this domain when compared with other SEUSS-LIKE plant proteins and animal Ldb proteins (Fig. 7A,B). Protein secondary structure prediction indicates that this domain of SEU likely forms an α helix (Fig. 7B). In addition, four hydrophobic residues spaced 7 residues apart within this region (Fig. 7B) suggest that this region may form a hydrophobic zipper.

While the Ldb proteins are similar to SEU only in the conserved central domain, the SEUSS-LIKE proteins from plants are similar to SEU in the entire protein. Arabidopsis genome has two SEUSS-LIKE genes, M7J2.110 (CAA18174) and MTG10.12 (BAB10171); both are 55% identical to SEU in the putative dimerization domain and 33% identical over the entire protein (Fig. 7A). A rice gene (AAF34437) has an overall 48% identity to SEU and a 81% identity in the putative dimerization domain (Fig. 7A). A second rice gene (BAA90807) is more closely related to the Arabidopsis M7J2.110 and MTG10.12 than it is to SEU. A large number of EST sequences from other plant species such as Gossypium arboreum (BG442742), Zea mays (AW066123), Lycopersicon esculentum (AW031470), Glycine max (AF100167), and Pinus taeda (AW043184) also share high levels of sequence similarity with SEU. Because of limited sequence information from these EST clones, only portions of these genes can be compared with SEU. Between 48% and 85% sequence identity within the dimerization domain is found among these SEUSS-LIKE plant proteins. However, the function of these SEUSS-LIKE genes is unknown.

DISCUSSION

SEU, together with LUG and AP2, regulates the spatial and temporal pattern of AG expression

We report the identification and characterization of a novel mutant seu in flower development. We showed that the partial homeotic transformation of floral organ identity and a slight reduction of floral organ number in seu single mutants are caused by ectopic and precocious AG expression. The sepal/petal and sepal/stamen mosaics observed in seu whorl 1 organs also suggest an ectopic B activity. However, this ectopic B activity may be mediated by the ectopic AG activity because ag seu double mutants no longer exhibited such sepal/petal or
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Hence, SEU is mainly involved in the negative regulation of AG in flowers. The relative weak phenotype of seu single mutants could be explained by several alternative but non-exclusive hypotheses. First, SEU may encode a co-regulator of LUG. In the presence of intact LUG, a defective SEU may only slightly reduce the activity of the LUG-SEU complex. Second, none of our seu alleles is a null as the truncated SEU protein in seu-1 or seu-2 might still possess partial function. Third, SEU may encode a member of a gene family whose function may be partially redundant with other family members.

The effects of seu mutations are most striking when combined with lug. More complete homeotic transformation in floral organs and a greater extent of floral organ loss are observed in the seu lug double mutants and are shown to be mediated by enhanced AG mis-expression. In particular, precocious AG expression was observed at stages as early as stage 1 and even pre-stage 1 in seu-1 lug-8 double mutant flowers. This stage 1/pre-stage 1 expression of AG was never observed in seu-1, lug-1, and ap2-2 single mutants, which cause precocious AG expression starting at stage 2 floral meristems (Drews et al., 1991; Liu and Meyerowitz, 1995; Liu et al., 2000). The stage 1/pre-stage 1 AG expression may underlie the dramatic reduction of floral organ number in the seu lug double mutants. Increased AG activity is known to repress floral organ initiation, particularly in whorls 2-3, and...

Fig. 7. Sequence similarity between SEU, SEUSS-LIKE proteins and Ldb proteins. (A) Diagramatic representation of the open reading frames of SEU, SEUSS-LIKE proteins from Oleracea sativa (AAF34437) and A. thaliana (CAA18174), representative Ldb family members from Danio rerio (AF031377), and Mus musculus (AF024524) and a putative Ldb family member from Saccharomyces pombe (AL031262). Numbers indicate amino acid residues. The shaded portion represents the putative dimerization domain for each protein. Percentages shown within the putative dimerization domain indicate amino acid sequence identity between SEU and the respective protein. Percentages shown to the right are percentage identity to SEU over the entire open reading frame. Glutamine-rich regions are shown as hatched boxes. LID: LIM interaction domain. (B) Sequence alignment of SEU with representative SEUSS-LIKE proteins, and Ldb family proteins in the putative dimerization domain. Identical residues are shaded black. Similar residues are shaded gray. The predicted alpha-helical portion of SEU is indicated with a two-headed arrow. Four hydrophobic or non-polar amino acids (+) in SEU are spaced seven residues apart in this region. Six hydrophobic or non-polar amino acids (+) in the M. musculus Ldb1 protein are also spaced seven residues apart, suggesting a hydrophobic zipper structure (Jurata and Gill, 1997).
AG was postulated to prevent organ primordial initiation by inhibiting cell proliferation (Bowman et al., 1991). In addition, dominant genetic interactions between seu-1 and ap2-2 and among seu-1, ap2-1 and lug-1 were also observed. Dominant genetic interactions have been reported previously between lug and strong ap2-9 (Liu and Meyerowitz, 1995) and may indicate direct physical interactions or a common activity threshold among the interacting proteins.

**SEU regulates other developmental processes independently of AG**

Both LUG and AP2 have functions that are independent of AG. AP2 specifies sepal and petal identity, while LUG regulates floral organ and leaf shape and gynoecium and ovule development (Bowman et al., 1991; Liu and Meyerowitz, 1995; Roe et al., 1997; Schneitz et al., 1997; Chen et al., 2000; Liu et al., 2000). With the exception of defects in floral organ identity and organ number, many of the seu defects are not suppressed by removing AG activity. For example, seu ag double mutants still display reduced plant height and form narrow sepals and petals. In addition, in seu lug double mutants, a small mound of tissue develops in whorl 4 (Fig. 2B-D). This reduced whorl 4 phenotype appears AG-independent as the seu lug triple mutants have a much reduced number of whorl 4 organs in the indeterminate flower. Finally, the seu lug ag triple mutants develop canoe-shaped sepals and blade-or club-shaped petals (Fig. 4C,E,F), suggesting a synergistic interaction between seu and lug in regulating organ shape. Hence, in addition to repressing AG, SEU, together with LUG, may regulate additional target genes that determine plant height, organ shape and whorl 4 primordium formation.

What underlies these AG-independent defects of seu? Examination of petal cells in seu single and seu lug ag triple mutants by scanning electron microscopy indicated that the petal cell size is similar to that of wild type (R.G. F. and Z. L., unpublished data). Hence, the narrow organ shape, reduced plant height, and reduced whorl 4 organ primordia are consistent with a general reduction of cell number, and, perhaps, reflect a role of SEU in promoting cell proliferation. LUG was similarly proposed to have such a role in cell proliferation control (Liu et al., 2000).

**SEUSS encodes a putative transcriptional co-repressor**

seu mutants are similar to lug mutants in their phenotype, their synergistic and dominant interaction with ap2, their ability to negatively regulate AG, and their role in regulating organ shape and gynoecium development. These similarities suggest that SEU may function similarly to LUG. We showed that SEU does not encode a protein with significant sequence similarity to LUG. Rather, SEU encodes a Q-rich protein with a conserved domain that is similar to the dimerization domain of Ldb family of transcriptional co-regulators. Our finding that both the seu-1 and seu-2 mutation results in the truncation of the SEU protein in this conserved domain suggests that this domain is important for SEU function.

Ldb protein family members regulate transcription via direct physical interactions with DNA-binding transcription factors such as the LIM-homeodomain proteins (Agulnick et al., 1996; Bach et al., 1997; Jurata and Gill, 1997). In the *M. musculus* Ldb1 protein, the domain conserved between Ldb1 and SEU was predicted to form an amphipathic α helix and mediate homo-dimerization (Jurata and Gill, 1997). In addition, Ldb proteins contain a second domain, the LIM Interaction Domain (LID) (Fig. 7A), which mediates the interaction between Ldb proteins and the LIM-homeodomain proteins. However, there is no sequence similarity in the LID domain between SEU and members of the Ldb family.

SEUSS-LIKE genes are found in *Arabidopsis*, rice, soybean, corn, pine and other plant species and define a novel family of plant regulatory proteins. With the exception of SEU, the function of other family members is not known. Our genetic and molecular analysis of seu is beginning to shed light on the function of this novel family of plant regulators. Furthermore, using a reverse genetic approach, we will be able to test whether the two *Arabidopsis* SEUSS-LIKE genes play redundant roles with SEU.

**A proposed model**

Based on our genetic and molecular analyses, we propose that SEU is a co-regulator of LUG. The domain structure of LUG is similar to that of a class of functionally related transcriptional co-repressors including Tup1 of yeast, Groucho of *Drosophila* and TLE (Transducin-like Enhancer of split) in mammals (Hartley et al., 1988; Williams and Trumbly, 1990; Parkhurst, 1998; Conner and Lii, 2000). In yeast, the Tup1 co-repressor is brought to target promoters by sequence-specific DNA-binding proteins and regulates a wide array of independent sets of genes such as a-cell specific genes, glucose-repressed genes, flocculation genes, and DNA-damage-induced genes (Roth, 1995; Teunissen et al., 1995). The N-terminal portion of Tup1 forms a repression complex with Ssn6, a tetratricopeptide repeat protein (Keleher et al., 1992), which is needed to facilitate the interaction between Tup1 and the corresponding DNA-binding transcription factors (Tzamarias and Struhl, 1994).

If LUG acts via a mechanism similar to Tup1, could SEU be the *Arabidopsis* equivalent of Ssn6? Although SEU does not share sequence similarity with Ssn6, both SEU and Ssn6 possess Q-rich domains, lack a DNA-binding motif, and contain a putative protein-protein interaction domain. Both seu and ssn6 mutants are pleiotropic in phenotype. The distinct molecular identity but similar genetic function between SEU and LUG also support that SEU and LUG may work together by forming a co-repressor complex. Preliminary experiments indicate that SEU interacts with LUG in the yeast two-hybrid system (A. Surendra Rao, R. G. F. and Z. L., unpublished data). Hence, our current working model predicts that by interacting with DNA-binding transcription factors that bind to AG cis-elements, the putative SEU/LUG co-repression complexes are recruited to repress AG expression in the outer two whorls of a flower. Candidate DNA-binding factors include, but are not limited to, AP2. This model could explain the synergistic and dominant genetic interactions among ap2, seu and lug. The molecular isolation of LUG (Conner and Liu, 2000), AP2 (Jofuku et al., 1994), and SEU (reported here) will allow us to further test these hypotheses. Other molecular and biochemical analyses will increase our understanding of the transcriptional repression mechanism in higher plants.

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