**SEUSS and AINTEGUMENTA Mediate Patterning and Ovule Initiation during Gynoecium Medial Domain Development**\(^1\)\(^{[W]}\)\(^{[OA]}\)

Sridevi Azhakanandam\(^2\), Staci Nole-Wilson\(^2\), Fang Bao, and Robert G. Franks\(^*\)

Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695

The Arabidopsis (Arabidopsis thaliana) gynoecium, the female floral reproductive structure, requires the action of genes that specify positional identities during its development to generate an organ competent for seed development and dispersal. Early in gynoecial development, patterning events divide the primordium into distinct domains that will give rise to specific tissues and organs. The medial domain of the gynoecium gives rise to the ovules, and several other structures critical for reproductive competence. Here we report a synergistic genetic interaction between seuss and aintegumenta mutants resulting in a complete loss of ovule initiation and a reduction of the structures derived from the medial domain. We show that patterning events are disrupted early in the development of the seuss aintegumenta gynoecia and we identify PHABULOSA (PHB), REVOLUTA, and CRABS CLAW (CRC) as potential downstream targets of SEUSS (SEU) and AINTEGUMENTA (ANT) regulation. Our genetic data suggest that SEU additionally functions in pathways that are partially redundant and parallel to PHB, CRC, and ANT. Thus, SEU and ANT are part of a complex and robust molecular system that coordinates patterning cues and cellular proliferation along the three positional axes of the developing gynoecium.

---

\(^1\) This work was supported by the National Research Initiative of the U.S. Department of Agriculture Cooperative State Research, Education, and Extension Service (grant no. 2006-03378 to S.N.W.), the National Science Foundation (grant no. IOB–0416759 to R.G.F.), and the U.S. Department of Agriculture Agricultural Research Service (grant no. 2006–03378 to S.N.W.).

\(^2\) These authors contributed equally to the article.

\(^*\) Corresponding author; e-mail rgfranks@ncsu.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Robert G. Franks (rgfranks@ncsu.edu).

\(^{[W]}\) The online version of this article contains Web-only data.

\(^{[OA]}\) Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.107.114751


Downloaded from on July 20, 2017 - Published by www.plantphysiol.org

Copyright © 2008 American Society of Plant Biologists. All rights reserved.
Figure 1. seu ant double mutants display enhanced vegetative, floral, and gynoecial phenotypes. A to C, False-colored photomicrographs. D to K, Photomicrographs of floral/rosette morphology; some sepals and petals have been removed from front half of flowers to allow viewing of gynoecium. L and M, Alcian-blue-stained gynoecial cross sections; N and O, Longitudinal optical section of cleared gynoecia (Nomarski optics). Scale bars: D to J, 1 mm; K, 10 mm; L to O, 0.1 mm. Asterisks (*) in M and O indicate expected location of ovules. A and B, Gynoecial cross sections at level of ovary from stage 8 (A) and stage 12 (B) flowers. Medial and lateral domains are show subdivided into adaxial (inner) and abaxial (outer) portions by dotted oval. Two medial ridges (mr) arise on adaxial portions of medial domain (purple/magenta) at stage 8 and give rise to ovules (ov) and septum (s) in mature gynoecium. The abaxial replum (abr) forms from the abaxial portion of the medial domain (orange). The lateral domains gives rise to carpel valves (cv). C, Side view of mature gynoecium indicates patterning elements along apical basal axis: stigma (stg), style (sty), and ovary (ovy). D, Wild-type Col-0; E, ant-3 mutant; F, ant-1 mutant, petals narrower than wild type (arrowhead); G, seu-3 mutant; H, seu-3 ant-3 double mutant displays enhanced carpel splitting at apex (arrowhead); I and J, seu-3 ant-1 double mutant displays filamentous petals (fp) and very reduced stamens (st); gynoecium (g) is split open and does not display ovules; medial domain displays small amounts of stigmatic (stg) and stylar tissue. K, Rosette phenotypes of Col-0 (left) and seu-3 ant-1 (right). L, In Col-0 gynoecium transmitting tract (tt) stains blue and ovules (ov) are indicated. M, Transmitting tract stains blue in the seu-3 ant-1 double mutant whereas ovules are missing. N, Developing ovules (ov) are observed attached to septum (s) in the seu-3 ant-3 double mutant. O, In the seu-3 ant-1 double mutant, no ovules grow out from septum (s).
provide two partially redundant, but parallel activities required for the development of the medial ridge and subsequent ovule initiation. Our results demonstrate that adaxial/inner identity is compromised early during the development of the gynoecium in seu ant mutants. We identify PHB, REVOLUTA (REV), and CRABS CLAW (CRC) as potential downstream targets of SEU and ANT regulation. In addition to the proposed linear regulatory relationship between SEU and the downstream targets PHB, REV, and CRC, our genetic analysis argues for an additional action of SEU in partially redundant pathway(s) parallel to PHB, CRC, and ANT.

RESULTS

seu ant Mutants Display Synergistic Floral and Vegetative Phenotypes

The seu single mutant has reduced stem length and shorter, rounder leaves (Franks et al., 2002). The seu plants also display multiple floral defects including partial homeotic transformations in perianth organs, narrower floral organs, reduction in stamen height and pollen production, partial splitting of the gynoeccial apex, and ovule defects. Additionally, the seu plants display phenotypes associated with a decreased response to auxin, including decreased apical dominance, fewer lateral root primordia, and a reduced sensitivity to exogenous auxin (Pfluger and Zambrayski, 2004). The ant single mutants display narrow floral organs, occasionally split carpel tips, ovule defects, slightly narrower leaves with reduced blade on the petiole section, and stamen locule defects (Elliott et al., 1996; Klucher et al., 1996).

To better understand the relationship of SEU and ANT, we generated seu antegumenta double-mutant plants. Organ counts in mature flowers revealed a synergistic role for SEU and ANT in the regulation of organ number in whorls 1 and 2 and in the control of organ size in all four whorls. The number of whorl-1 organs was significantly reduced in the seu ant double mutant, relative to wild type (Table I). Nearly all whorl-1 organs developed as narrow sepals (Fig. 2C), and no enhancement of homeotic transformation was detected. This is in contrast to whorl-1 organs in previously described lug ant flowers that typically display homeotic carpelloid transformations (Liu et al., 2000). The number of organs in whorl 2 was also significantly reduced in the seu ant mutant relative to wild type (Table I). In seu-3 ant-1 mutants, whorl-2 organs developed as small radialized filamentous structures that failed to display cellular characteristics of mature petals (Fig. 1I; data not shown). Weaker allelic combinations employing the ant-3 weak hypomorphic allele (Klucher et al., 1996) also displayed synergistic enhancement with respect to organ loss and organ shape and size (Fig. 1H). Scanning electron microscopy (SEM) analysis indicated that reduced numbers of floral organs resulted from early failure to initiate these organs (Fig. 2). In contrast to whorls 1 and 2, the effects of seu and ant on organ number in whorl 3 were additive (i.e. the number of stamens in the seu-3 ant-1 double was similar to that in the ant-1 single). Thus, most of the loss of stamens can be accounted for by the loss of ANT activity. The anthers of ant mutant stamens comprise just two locules compared to four locules in the wild-type anther (data not shown). The anther defect is further enhanced in the seu-3 ant-1 mutant resulting in complete male sterility (Figs. 1, I and J, and 2, I and K). Carpel number was unchanged in all mutant combinations examined (Table I). However, the average length of mature stage-12 gynoecia (stages according to Smyth et al., 1990) from seu-3 ant-1 mutants was statistically shorter than ecotype Columbia of Arabidopsis (Col-0) and either single mutant. The enhancement of carpel morphology observed in the seu ant double mutant is further described in the following section.

The rosette leaves of the seu ant mutants are shorter and narrower than those of either single mutant or

### Table 1. Morphometric and phenotypic analysis of seu, ant, and seu ant mutant plants

<table>
<thead>
<tr>
<th></th>
<th>Col-0</th>
<th>Col-gl</th>
<th>ant-3</th>
<th>ant-1</th>
<th>seu-3</th>
<th>seu-3 ant-1</th>
<th>seu-3 ant-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ no. whorl 1</td>
<td>4.0 ± 0.0</td>
<td>ND*</td>
<td>ND</td>
<td>3.9 ± 0.25</td>
<td>4.0 ± 0.0</td>
<td>2.6 ± 0.67b</td>
<td>ND</td>
</tr>
<tr>
<td>n = 20</td>
<td></td>
<td></td>
<td></td>
<td>n = 30</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organ no. whorl 2</td>
<td>4.0 ± 0.0</td>
<td>ND</td>
<td>ND</td>
<td>3.97 ± 0.18</td>
<td>4.0 ± 0.0</td>
<td>2.9 ± 1.01b</td>
<td>ND</td>
</tr>
<tr>
<td>n = 20</td>
<td></td>
<td></td>
<td></td>
<td>n = 30</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organ no. whorl 3</td>
<td>5.9 ± 0.05</td>
<td>ND</td>
<td>ND</td>
<td>4.2 ± 0.50b</td>
<td>5.6 ± 0.62</td>
<td>3.7 ± 0.55b</td>
<td>ND</td>
</tr>
<tr>
<td>n = 20</td>
<td></td>
<td></td>
<td></td>
<td>n = 30</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organ no. whorl 4</td>
<td>2.0 ± 0.0</td>
<td>ND</td>
<td>ND</td>
<td>2.0 ± 0.0</td>
<td>2.0 ± 0.0</td>
<td>2.0 ± 0.0</td>
<td>ND</td>
</tr>
<tr>
<td>n = 20</td>
<td></td>
<td></td>
<td></td>
<td>n = 30</td>
<td>n = 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average carpel length</td>
<td>2.2 ± 0.12</td>
<td>ND</td>
<td>ND</td>
<td>2.1 ± 0.17</td>
<td>2.0 ± 0.19</td>
<td>1.5 ± 0.22b</td>
<td>ND</td>
</tr>
<tr>
<td>(in millimeters)</td>
<td>n = 10</td>
<td>n = 9</td>
<td>n = 10</td>
<td>n = 10</td>
<td>n = 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average ovules per</td>
<td>25 ± 2.0</td>
<td>21 ± 3.0</td>
<td>20 ± 2.7</td>
<td>12 ± 1.3b</td>
<td>23 ± 1.8</td>
<td>0.0 ± 0.0b</td>
<td>13 ± 3.4b</td>
</tr>
<tr>
<td>carpel</td>
<td>n = 16</td>
<td>n = 22</td>
<td>n = 28</td>
<td>n = 24</td>
<td>n = 12</td>
<td>n = 18</td>
<td>n = 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent of ovules</td>
<td>0% n = 76</td>
<td>ND</td>
<td>13% n = 30</td>
<td>ND</td>
<td>22% n = 106</td>
<td>ND</td>
<td>76% n = 45</td>
</tr>
<tr>
<td>missing female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gametophyte</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*ND, Not determined. bIndicates a statistically significant difference when compared to wild-type reference allele using Student’s t test at P < 0.001.
wild type (Fig. 1K). These shape changes are sometimes associated with a reduction in abaxial or adaxial fate assignment in the leaf (Waites and Hudson, 1995). To determine if the rosette leaves displayed adaxial or abaxial fate alterations, we examined epidermal cell morphology in the leaf blade by SEM. In wild-type rosette leaves, the leaf blade cells on both the adaxial and abaxial surfaces resemble jigsaw puzzle pieces. The cells of the adaxial surface are larger, more uniform in size, and less lobed than those of the abaxial surface (McConnell and Barton, 1998; Fig. 2, L and M). Additionally, the adaxial blade surface is flatter than

the abaxial blade surface; the later is more undulating. In seu-3 ant-1 rosette leaves the adaxial leaf epidermal cells were slightly more lobed, more variable in size, and the leaf surface was more undulating when compared to wild type. These phenotypes suggest a partial loss of adaxial identity in seu-3 ant-1 leaves (Fig. 2O). A similar but weaker effect was observed on the adaxial surface of the seu-3 leaf (Fig. 2N). The edges of seu-3 adaxial leaf cells appeared more lobed and the adaxial surface was more undulating compared to the flat adaxial surface of wild-type leaves. No alterations of epidermal cell morphology were observed on the abaxial leaf surfaces (data not shown). In addition to leaf defects, seu ant double mutants exhibited more severe internode elongation defects than either single mutant resulting in a semidwarf phenotype (data not shown).

**seu ant** Mutants Display Early Gynoecial Defects and Lack Ovule Primordia

To determine the effect of seu and ant on ovule initiation, we counted ovules from chloral hydrate-cleared stage-10 gynoecia. The average number of ovules observed in the seu-3 single mutants was not statistically different from wild type (Table I). However, the ant-1 single mutants averaged half the wild-type number of ovules. Notably, the seu ant double mutants lack ovules completely. Analysis of the double mutants by thin section analysis, SEM, and chloral hydrate clearing confirmed the complete loss of ovule primordia initiation (Figs. 1, L–O, and 2K). The seu ant double-mutant gynoecia, although split apart toward the apex, were fused normally in basal portions of the gynoecium. In these fused areas abaxial replum and some septal tissue formed (Fig. 1M). Alcian blue staining of cross sections was used to detect transmitting tract cells (Sessions and Zambryski, 1995). In Col-0, ant-1, and seu-3 gynoecia, transmitting tract cells could be detected in all sections examined after stage 12 (Fig. 1L). In the seu-3 ant-1 double mutants, transmitting tract cells could be detected in about 50% of the appropriately staged gynoecia (Fig. 1M). In many cases the extent of transmitting tract was reduced relative to the single mutants or wild type. In the other 50% of instances no transmitting tract was detected. Development of the stigmatic and stylar tissues was reduced in the seu-3 ant-1 double mutant, but was detected (Fig. 1I). In comparison to the gynoecial phenotypes reported for the lug ant double mutant, the seu ant phenotypes are less severe. The seu ant gynoecia nearly always displayed stigma, style, and septum (albeit reduced), whereas lug ant gynoecia did not display stigmatic or stylar tissues, and only exhibited partial septum development when intermediate-strength lug alleles were examined (Liu et al., 2000). Furthermore, the degree carpel fusion is greater in the seu ant gynoecia, with component carpels nearly always fused together in the bottom third of the gynoecium, whereas the lug ant gynoecia are only infrequently fused to this degree. This is unlikely to be due to allele-specific or ecotype-specific effects because seu-1 ant-9 double mutants in the Ler ecotype display a phenotype that is similar to the seu-3 ant-1 Col-0 ecotype plants reported here (R.G. Franks, unpublished data).

Weaker allelic combinations employing the ant-3 hypomorphic allele (Klucher et al., 1996) also had synergistic ovule initiation defects. The average number of ovules in ant-3 plants was not significantly different from the reference ecotype Col-glabra (Col-gl; Table I). However, the seu-3 ant-3 plants initiated significantly fewer ovules per carpel than either single mutant. The seu-3 ant-3 double mutants also displayed enhanced splitting of the carpels (Fig. 1H). We examined mature, chloral hydrate-cleared seu-3 ant-3 ovules and found no evidence of female gametophyte development in 76% of these ovules (Table I; Fig. 2, R–U). The other 24% of the seu-3 ant-3 ovules displayed incompletely developed female gametophytes that appeared stalled at various stages of development. The ant-3 and seu-3 single-mutant ovules also displayed disrupted female gametophyte development, albeit less frequently than was observed in the double mutant (Table I). The extent of growth of the outer integument in seu-3 and both the outer and inner integuments in ant-3 was reduced relative to wild type (Fig. 2, R–U). This disruption of integument growth was more frequent and more pronounced in the seu-3 ant-3 ovules than in the seu-3 or ant-3 single mutant. However, integument disruptions in the seu-3 ant-3 ovules were less pronounced than in the strong loss-of-function ant-1 single mutant that displays almost no integement development (Klucher et al., 1996).

We examined early stages of floral and gynoecial development in the seu, ant, and seu ant flowers with SEM to determine when morphological defects were first observed. The earliest morphological defect we observed in seu ant mutant floral meristems occurred at floral stage 3. In wild-type stage-3 flowers, four sepal primordia arise on the flanks of the floral meristem in whorl 1 (Fig. 2A). The seu-3 and ant-1 single mutants typically also displayed this morphology (data not shown). However in the seu ant double-mutant flowers, often only two or three sepal primordia were observed suggesting an early defect in organ initiation (Fig. 2, B and C).

Gynoecial development in wild-type flowers initiates at stage 6 as the gynoecial tube arises from the remaining portion of the floral meristem. In all genotypes we examined, including the seu ant double mutant, the gynoecial tube arose normally (Fig. 2, D–G). Shortly after the gynoecial tube forms, the medial ridges are observed as two distinct bulges on the adaxial (inner) surface of the tube in a medial position (Fig. 2D). These medial ridges will give rise to the placental tissue and ovule primordia, as well as septum and transmitting tract tissue. Development of gynoecia from seu or ant single-mutant flowers appears morphologically indistinguishable from wild type at this stage (Fig. 2, E and F). In some seu ant
gynoecia the early development of the medial ridge appears wild type. In other cases it appears to be reduced in its extent at this early stage of development, but still morphologically distinguishable (Fig. 2G). During stages 7 and 8, the gynoecial tube begins to elongate. In wild-type gynoecia, as well as in the seu and ant single mutants, this elongation is coordinated with respect to the medial and lateral domains ensuring a fused tube grows up evenly (Fig. 2H; data not shown). In the seu ant double mutants, splitting of the gynoecial apex results from the separation of the medial and lateral domains (Fig. 2I). Growth within the medial domains is retarded relative to the lateral domains and extended horn-like structures develop from the lateral domains in later stages (Fig. 2K).

In mature wild-type gynoecia the vascular bundles extend throughout the basal to apical extent of the ovary within the medial and the lateral domains (Kuusk et al., 2002). The lateral vascular bundle terminates at the distal portion of the ovary at the junction of the ovary and the style. The medial vascular bundle extends into the stylar tissue and terminates in a fan-shaped vascular array. Development of the medial and lateral vascular bundles was similar to wild type in the seu-3 ant-3 double mutant (containing the hypomorphic ant-3 allele; Fig. 2P, arrowhead). However, in the seu-3 ant-1 double mutants, vasculature development in the two medial domains was retarded relative to that in wild-type and vascular bundles consistently terminated within the basal third of the gynoecium with no apparent development of the terminal vascular array (Fig. 2Q, arrowhead). The development of the lateral vascular bundles in the seu-3 ant-1 double mutants was indistinguishable from wild type and terminated in the apex of the lateral horns (Fig. 2Q, arrow; data not shown).

**seu ant** Gynoecia Fail to Maintain Expression of Adaxial Fate Regulators **PHB and REV**

PHB, PHAVOLUTA (PHV), and REV are expressed within and are required for the specification of adaxial domains of Arabidopsis lateral organs (Zhong and Ye, 1999; Eshed et al., 2001; McConnell et al., 2001; Otsuga et al., 2001; Prigge et al., 2005). Additionally, they are expressed within adaxial portions of the gynoecium and are likely important for the specification of adaxial fate during gynoecial development (Otsuga et al., 2001; Prigge et al., 2005). As SEU and ANT regulate the expression of the polarity genes **YAB1/FIL** and **PHB** in petals and in leaves (Franks et al., 2006; Nole-Wilson and Krizek, 2006). At stage 5, the stamen primordia begin to separate from the future gynoecium (gynoecial anlagen), and **PHB** expression is detected in the core of the gynoecial anlagen as well as within the core region of the stamens (arrows); expression within the adaxial portions of the sepal is also detected (Fig. 3B). During stage 6, **PHB** expression continues to be detected within the core of the gynoecium and becomes more restricted within the stamen primordia to adaxial portions (Fig. 3, C and D). During stage 7, the expression domain in the gynoecial core resolves to two more lateral domains that likely mark the adaxial portions of the valve domains (Fig. 3E, arrowheads). Expression within the stamens is detected most strongly in an arc that may mark the boundary between adaxial and abaxial domains (Fig. 3E, arrow). The expression of **PHB** is strongest along this arc within marginal portions of the stamen. At later stages expression is detected in the placenta and early ovule primordia (Fig. 3F) and continues to be expressed in portions of the ovules (Sieber et al., 2004).

**PHB** expression within seu mutant flowers was indistinguishable from wild type at all stages examined (data not shown). Expression of **PHB** within ant mutant stage-3 flowers was detected in a pattern similar to that of stage-3 wild-type flowers (Fig. 3G). However, from stage 5 onward, the expression of **PHB** was weaker overall in the ant mutant flowers (Fig. 3, H–J). The reduced expression was more severe in the gynoecium than in the stamen primordia in which near wild-type levels of expression were typically detected (Fig. 3I, arrow). This molecular phenotype was not completely penetrant. However, we observed a loss or reduction of **PHB** signal during stages 5 or 6 in more than half of the ant mutant gynoecia examined. To control for variations in staining from experiment to experiment, we judged expression levels of **PHB** relative to expression levels in stage-3 flowers from the same in situ hybridization slide. In later stage gynoecia, stage 10 and older, **PHB** expression was strongly reduced or not detected in the ant-1 gynoecium (Fig. 3J) although occasional exceptions with intermediate levels of staining were noted.

Within the seu ant double mutants, expression of **PHB** in stages 1 to 4 was indistinguishable from wild type (Fig. 3K). However, again we detected a reduction of expression within the adaxial core of the gynoecium at stages 5 and 6 that was more obvious in the seu ant double mutant than in the ant single mutant (compare Fig. 3L with Fig. 3D). This phenotype was also not fully penetrant at this stage, but was observed in more than half of the gynoecia examined. Expression of **PHB** was not detected in seu ant gynoecia older than stage 9 (Fig. 3M) except in vasculature tissues (data not shown). These results suggest that **SEU** and **ANT** play a role in the maintenance of expression of **PHB** expression during the transition of the floral meristem into the gynoecial primordia and at later stages of gynoecial development. These alterations in expression pattern, particularly those at stages 5 and 6,
Figure 3. Adaxial fate determinants PHB and REV in seu ant gynoecia. In situ hybridization with PHB antisense (A–M) or REV antisense (N–T) probes. A, D, F, G, J, K, L, and M, Floral longitudinal sections. B, C, E, H, I, and N to T, Floral cross sections (extent of gynoecium encircled by black dashed ovals). All scale bars are 0.1 mm. Numbers refer to floral stages. A, PHB expression detected in central core of stage-3 Col-0 flower. B and C, PHB expression detected in adaxial core of gynoecial (arrowhead) and stamen (arrow) anlagen/primordia. D, PHB expression in gynoecium (arrowhead). E, PHB expression in adaxial valve domains of gynoecium (arrowheads). F, PHB expression in placenta and ovule primordia (arrowhead). G, PHB expression detected in central core of ant-1 floral meristem. H and I, PHB expression is reduced or not detected in ant-1 gynoecia (arrowheads). Expression in
preceded the earliest morphological alterations that are observed in seu ant gynoecia, suggesting that the loss of PHB expression is not simply due to a loss of the medial ridge precursors.

REV is also expressed within the adaxial core of early floral primordia, as well as the core of the gynoecial primordia (Otsuga et al., 2001; Prigge et al., 2005). REV continues to be expressed in adaxial portions of the gynoecium during stages 6 through 11 (Fig. 3, N–P). In seu and ant single mutants, expression of REV was indistinguishable from wild type (data not shown). In the seu ant double mutant, REV expression is strongly detected in the adaxial core of gynoecia (stages 6 and 7) at levels similar to wild type (Fig. 3, Q and R). By stages 8 and 9, expression within the adaxial gynoecium appeared reduced relative to wild type, however, weak expression was detected in adaxial portions of the gynoecium suggesting that adaxial fate is partially maintained in these double mutants (Fig. 3, S and T).

Quantitative real-time PCR (qRT-PCR) analysis confirmed the in situ hybridization studies. We examined expression levels from two different RNA sources: inflorescence apex (i.e. inflorescence meristem through floral stage 6) and dissected gynoecia from flowers (stages 8–10; Fig. 3, U and V). In the inflorescence apex, PHB levels in the seu-3 and seu-3 ant-1 genotypes were significantly reduced ($P < 0.001$) to 68% and 29% of wild-type levels, respectively. REV levels in the inflorescence apex were not significantly different from wild type in the seu or ant single mutants. In the seu-3 ant-1 double mutant, REV levels did display a significant reduction compared to wild type. Consistent with our in situ hybridization data, the magnitude of this reduction was less than that observed for PHB. In the dissected gynoecia (stages 8–10), PHB levels in the seu-3 and ant-1 genotypes displayed a signficantly ($P < 0.05$) reduction. The levels of PHB in the seu ant double-mutant gynoecia were further reduced to 12% of wild type ($P < 0.001$). Levels of REV in the dissected gynoecia also displayed highly significant reductions in the ant-1 and seu-3 ant-1 genotypes.

**seu ant** Gynoecia Display Altered Expression of Abaxial Fate Determinant CRC

The reduced levels of PHB and REV expression in the seu ant mutant suggest that adaxial identities may be compromised. Because of an antagonistic regulatory interaction between adaxial and abaxial fates in lateral organs, a reduction in adaxial fate is often accompanied by an ectopic expansion of abaxial fate (Bowman et al., 2002). To determine if abaxial fate was expanded in seu ant mutant carpels, we examined the expression of the gynoecial abaxial fate determinants YAB1/FIL and CRC by in situ hybridization. Both CRC and YAB1/FIL are members of the YABBY family of putative transcriptional regulators (Bowman and Smyth, 1999; Chen et al., 1999; Sawa et al., 1999; Siegfried et al., 1999). Genetic analysis indicates that CRC plays a role in the maintenance of abaxial fate in the medial domain of the carpel, a function that is partially redundant with KANADI and GYMNOS activities (Eshed et al., 1999).

In wild-type gynoecia CRC expression initiates during late stage 5 or early stage 6 within the abaxial valve domains (Fig. 4A; Bowman and Smyth, 1999). During late stage 7, expression is detected in a ring that includes the abaxial-most one or two cell layers of both the valve and margin domains (Fig. 4B, arrow). Expression in the abaxial epidermis continues in the margin domain through stage 10 and in the lateral domain into stage 11 (data not shown). During stage 7, CRC is also expressed within an internal expression domain that initiates as two stripes that appear to mark the boundary between the valve and margin domains (Fig. 4B, arrowheads; Bowman and Smyth, 1999). The internal expression domain may be important for ovule initiation or development (Bowman and Smyth, 1999; Alvarez and Smyth, 2002). During stage 8, the internal expression domain consists of the adaxial valve epidermal cell layer (Fig. 4C, asterisk) and four subepidermal foci (Fig. 4C, arrowheads) located close to the sites of ovule initiation. These foci span the boundary between the medial and lateral domains and consist of between 15 and 20 CRC-positive cells in an 8-micron tissue section. A slightly different pattern of CRC is detected in apical portions of the gynoecium (within 10–15 microns of the apex). In the gynoecial apex (stages 7 and 8), CRC is expressed throughout most of the of the valve domain, and in the abaxial medial domain, but is not expressed in the adaxial portions of the medial domain (i.e. the medial ridge; Fig. 4D).

The seu and ant single mutants display wild-type patterns of CRC expression (data not shown). However, alterations of CRC expression were observed in the seu ant double mutants. Expression in stage-6 seu ant gynoecia initiates normally (data not shown). However, in seu ant gynoecia (stages 7 and 8), CRC expression within internal domains was very reduced or absent whereas expression in the abaxial epidermis...
was still strongly detected. This was most clearly exhibited in the basal third of the gynoecium in which the internal expression domain was often not detected (Fig. 4E). At midgynoecial sections (Fig. 4F, H and J) expression of CRC in the internal expression domain was detected, but expression was weaker and disor-
ganized. The expression of CRC in the adaxial valve epidermis was more consistently detected than expression within the subepidermal foci. Typically from zero to five CRC-positive cells were detected in these foci in the seu ant mutant. Occasionally the extent of the medial ridge was reduced (Fig. 4F, bottom half of the gynoecium) or missing (Fig. 4H, bottom half of the gynoecium). In the apical portion of the seu ant gynoecium, we detected ectopic expression of CRC within the median ridge. Although CRC is not normally expressed in the apical medial ridge in wild type (Fig. 4D), in the seu ant double mutants CRC was strongly detected in the medial ridge cells at the gynoecial apex (Fig. 4, G and K). These results indicate that CRC expression is differentially affected in the seu ant mutants along the apical/basal axis of the gynoecium. We note that ANT (Fig. 4L) and SEU (data not shown) are expressed in the medial domains at the apex of wild-type gynoecia and thus could be playing a direct role in the repression of CRC in these cells.

YAB1/FIL expression within the developing gynoecium is detected in abaxial subepidermal portions of the lateral domains that will later form the carpel valves (Fig 4M; Sawa et al., 1999; Siegfried et al., 1999). We also detected narrow rays or fingers of weaker expression that extend from the lateral domains into the portions of the medial domain that subtend the ovule primordia (Fig. 4, M, Q, R, and S). Serial cross sections indicated that these fingers were often, but not always, detected subtending sites of ovule primordium initiation. It appears that the cells of the YAB1/FIL-expressing fingers are a subset of the CRC-positive subepidermal foci cells, or closely abut them. YAB1/FIL expression in the seu and ant mutants appears to be unchanged relative to wild type. In the seu ant double-mutant YAB1/FIL is expressed normally in the abaxial valve domain, however, expression within the medial domain fingers was less consistently observed than in wild type (Fig. 4N). We also examined YAB1/FIL expression within the apex of the gynoecium. YAB1/FIL is not detected in the medial ridge at the gynoecial apex in wild-type gynoecia, nor in the seu, ant or seu ant mutants (Fig. 4, O and P). Thus, in contrast to CRC, YAB1/FIL is not ectopically expressed in the apical medial ridge in the seu ant double mutant.

SEU Protein Localizes to the Nucleus and Is Expressed Widely throughout the Plant

Northern analysis indicated that SEU RNA is expressed widely throughout all tissues examined (Franks et al., 2002). Examination of expression data available through the GENEVESTIGATOR site (Zimmermann et al., 2004) also suggest a widespread expression pattern for SEU RNA. SEU appears to play a role in determining expression of downstream target genes within domains of flower (repression of AG in perianth organ), but not reproductive structures; Franks et al., 2002; Sridhar et al., 2004, 2006) and domains of the gynoecium (this study). Thus, we wanted to examine the expression of the SEU protein during floral development to determine if localized expression of the SEU protein contributed to its specificity of action with respect to gene regulation. We generated C-terminal and N-terminal GFP translational fusion genomic rescue constructs: pSEU::GFP:SEU and pSEU::SEU:GFP. Three independent transformants were generated for each of the rescue constructs in a seu-1 mutant background and all rescued the aboveground defects of the seu-1 mutant (root phenotypes were not examined; data not shown). For all transformants, GFP florescence was detected in all tissues examined: root, young leaf, vegetative, and reproductive SAM, floral meristems, and all floral organs (Fig. 5; data not shown). GFP florescence was detected in the nucleoplasm, but was largely absent from the nucleolus and cytoplasm, or was detected at significantly reduced levels in these subcellular regions. Expression in the nucleoplasm is consistent with the predicted nuclear localization signal and the proposed role of SEU as a transcriptional coregulator (Franks et al., 2002). Within the developing

![Image](https://www.plantphysiol.org)
floral meristem (Fig. 5B) and gynoecium (Fig. 5C), GFP fluorescence was detected throughout all stages and subdomains. The widespread expression of SEU protein indicates that localized expression of SEU is not responsible for its domain-specific action.

**Genetic Interactions between *seu*, *crc*, and *ant***

Our in situ hybridization results indicate a complex relationship between *SEU*, *ANT*, and *CRC*. To better understand the regulatory relationships of these three genes, we analyzed *seu crc* double mutants and found that *seu* and * crc* displayed synergistic interactions with respect to carpel fusion and development of stigmatic tissues. In comparison to wild-type gynoecia, the * crc* mutant has a shorter and wider gynoecium that contains fewer ovules (Alvarez and Smyth, 1999, 2002; Fig. 6B). The * crc* gynoecium is also split within the apical-most third due to a failure of the two component carpels to maintain fusion. The *seu* single-mutant gynoecium often displays a small amount of splitting at the apex (Franks et al., 2002; Fig. 6C). This split is not between the two component carpels, but rather a split within the style or stigmatic tissue between the two fused marginal domains. The *seu crc* double-mutant gynoecium displays dramatically enhanced splitting between the two component carpels that extends for approximately 90% of the apical/basal extent of the gynoecium (Fig. 6D). The valves display horn-like extensions at the apex and there is an enhanced loss of stigmatic tissue. This synergistic enhancement between *seu* and * crc* is not found in *ant crc* double mutants that display a largely additive phenotype (Eshed et al., 1999; R.G. Franks, unpublished data).

**Can the *seu ant* Double-Mutant Phenotype Be Rescued by Supplemental *PHB* Activity?**

We sought to determine if the reduction of *PHB* expression in the *seu ant* double mutant was sufficient to explain the observed ovule loss. To test this, we attempted to rescue the *seu ant* carpel phenotype by replacing *PHB* expression with a 35S:*PHB* construct. A 35S:*PHB* expression construct (kindly provided by M. Prigge and S. Clark, University of Michigan, Ann Arbor, MI) that is functional to rescue the *phb phv cna athb8* mutant (Prigge et al., 2005) was crossed into the *seu ant* double mutant. Plants expressing the 35S:*PHB* construct are wild type in appearance, are self-fertile, and display normal ovule initiation and development (McConnell et al., 2001; Prigge et al., 2005) likely due to the targeted degradation of *PHB* transcripts by microRNAs 165/166 in abaxial domains (Emery et al., 2003). In all observable phenotypes the *seu ant* 35S:*PHB/+ plants were indistinguishable from the *seu ant* double mutants (data not shown). These results indicate that the 35S:*PHB* construct is not sufficient for rescue of the carpel defects of the *seu ant* mutant.

We additionally attempted to rescue the *seu ant* mutant with a *phb-1d* semidominant gain-of-function allele of *PHB* (McConnell and Barton, 1998; McConnell et al., 2001). To generate self-fertile *phb-1d* heterozygotes, plants were grown at 16°C (McConnell and Barton, 1998). The *phb-1d* allele generates a *PHB* transcript that is resistant to microRNA degradation and leads to the partial adaxialization of leaves, sepals, petals, and stamens (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003). The *phb-1d/+* mutant gynoecium also displays signs of adaxialization including the growth of ectopic ovules (adaxial structures) from the abaxial base of the gynoecium (McConnell and Barton, 1998; McConnell et al., 2001). However, adaxially (within the *phb-1d/+* gynoecium) the initiation and development of ovules progresses normally, thus supporting female self-fertility. The presence of ovule primordia within the *phb-1d/+* gynoecium allowed us to test genetic epistasis of the *phb-1d* allele with respect to the ovule-less *seu ant* mutant phenotype. We reasoned that the *phb-1d* allele might replace lost adaxial activity in the *seu ant* mutant and thus rescue ovule development. We also reasoned that if
SEU and ANT function upstream of PHB transcription that the loss of SEU and ANT might suppress or partially mitigate the *phb-1d* phenotype in the leaves and petals.

In the rosette leaves of the *phb-1d/+* heterozygotes grown at 16°C, the area of the leaf blade is reduced and the petiole is longer relative to wild type (Fig. 7B). The rosette leaf phenotype of the *phb-1d/phb-1d* homozygote is more severe. These rosette leaves display only small amounts of laminar expansion of the leaf blade, and trumpet-shaped leaves were observed (Fig. 7D). In contrast to our expectations, we observed enhanced radialization and narrowing of rosette leaves in the *phb-1d/+ seu ant* mutants (Fig. 7C) approaching that exhibited by the *phb-1d* homozygote. We also observed an enhancement of the *phb-1d* homozygous phenotype in *phb-1d seu* double (Fig. 7E) and *phb-1d seu ant* mutants (Fig. 7F). Thus, the loss of SEU, or SEU and ANT together, caused a further radialization and loss of laminar expansion of rosette leaves in the *phb-1d* homozygote background. In these plants almost all leaves were completely radialized and rod shaped. These plants also did not produce an observable inflorescence whereas the *phb-1d* homozygotes did.

Floral phenotypes also indicated that the *seu* and *ant* mutations enhanced (in petals) or were epistatic to (in gynoecia) the *phb-1d* mutation, rather than functioning as suppressors. Enhancement in the degree of radialization was observed in the petals of *phb-1d/+ seu* and *phb-1d/+ ant* mutants relative to the *phb-1d/+* petals (Fig. 7, J and K). With respect to the gynoecial phenotype, the *seu ant* double mutant was epistatic to the *phb-1d/+* mutant. The *phb-1d/+ seu ant* mutant gynoecia were very similar phenotypically to those of the *seu ant* double mutants in that medial domains formed but failed to generate ovule primordia (Fig. 7L).

**DISCUSSION**

ANT regulates organ initiation and organ size in part by maintaining the proliferative potential of organ primordia (Krizek, 1999; Mizukami and Fischer, 2000). More recent analysis of *ant fil* double mutants indicates that ANT, in concert with YAB1/FIL, is also involved in the regulation of floral organ identity and organ polarity along the abaxial/adaxial axis (Nole-Wilson and Krizek, 2006). SEU together with *LUG* has also been
shown to regulate cell number during organogenesis, as well as organ identity and organ polarity (Franks et al., 2002, 2006). Here we report synergistic interactions between the seu and ant mutants in both vegetative and reproductive tissues. Our results indicate that SEU and ANT share a number of partially redundant functions during Arabidopsis development. The seu ant gynoecia display a complete loss of ovule initiation and a reduction of the gynoecial medial domain. These morphological defects may in part be due to a reduction in adaxial identity within the gynoecial core as well as a reduction of cell proliferation or growth within the medial domain. We identify PHB, REV, and CRC as potential downstream targets of SEU and ANT regulation in the gynoecium. Additionally, we propose that SEU is required to support the activities of genes that function in parallel to ANT, PHB, and CRC during the development of the medial domain of the gynoecium.

Expression of PHB and REV Are Reduced in the seu ant Gynoecium

SEU in concert with LUG has been shown to function in the maintenance of PHB expression in petals (Franks et al., 2006). ANT in concert with YAB1/FIL is required for the expression of PHB in leaves and developing flowers (Nole-Wilson and Krizek, 2006). Our data indicate that the accumulation of PHB RNA in the seu ant mutants is significantly reduced relative to wild type. This effect is most strongly observed in the adaxial core of developing gynoecium during stage 6 and in the adaxial valves during stages 7 through 10. The analysis of REV RNA accumulation indicates that REV RNA is also significantly reduced in the seu ant mutants. REV expression in early stages 6 and 7 appeared at wild-type levels by in situ hybridization whereas at later stages REV expression was slightly, but significantly reduced (Fig. 3). The detection of REV within the adaxial core of the gynoecium through stage 7 indicates that the reduction of PHB levels in the seu ant double are not simply due to a loss of cells in this zone of the gynoecium. Thus we propose that one action of SEU and ANT is to support the expression or accumulation of PHB and REV in the developing gynoecial core and thus potentiate adaxial identity within the gynoecium. Alvarez and Smyth (2002) propose a model in which the expression of adaxial identity genes within the adaxial valve domain is required for development of the placenta within the juxtaposed medial domain. Our data are consistent with this model. However, we report previously undocumented expression of PHB and REV within the adaxial portion of both the medial and lateral domains of the stage-6 gynoecium (Fig. 3). Thus, another interpretation is that placental specification and subsequent ovule initiation requires the expression of adaxial identity genes within the medial domain itself. The molecular identities of SEU and ANT as transcriptional regulators suggest that the regulation of PHB and REV RNA accumulation may be direct and at the transcriptional level, however, we cannot exclude other possibilities. A sequence that matches the ANT binding site consensus in 12 of 14 conserved positions (allowing for a single base-pair gap) is located 1,335 bp upstream of the PHB translation start site (data not shown). However, Nole-Wilson and Krizek (2006) failed to detect significant binding of a bacterial expressed ANT protein to this template in a gel shift assay.

We used two independent methods to replace PHB activity in the seu ant mutant to determine if adding back PHB could rescue the ovule-less phenotype. We found that neither a 35S:PHB overexpression construct, nor the phb-1d allele provided rescue of the seu ant ovule loss (Fig. 7). These results suggest that the reduced expression of PHB is not likely sufficient, by itself, to explain loss of ovule primordia. SEU and ANT likely regulate additional genes during gynoecial development (e.g. REV and CRC) that may contribute to ovule initiation and medial domain development. An alternative, but not mutually exclusive, hypothesis is that SEU and ANT support cell division or growth within the medial domain independently from their role in the regulation of adaxial identity. The observation that seu and ant enhanced the leaf and petal defects of the phb-Yd plants indicates a role of SEU and ANT in parallel to PHB in the regulation of laminar growth.

Expression of CRC Is Regulated in Different Directions by SEU and ANT in Apical versus Ovarian Portions of the Gynoecium

SEU and ANT together cooperate to regulate the expression of CRC within the developing gynoecium. However, the effect of SEU and ANT on CRC expression appears to be different in the apex of the gynoecium than it is in the ovary (Fig. 4). Within ovarian portions of the gynoecium, SEU and ANT support the expression of CRC in internal domains. However, within the apex of the gynoecium, SEU and ANT repress the expression of CRC in the medial ridge. Thus, the action of SEU and ANT on CRC expression likely depends on additional factors or may be indirect. In support of the former, SEU shares homology to the LIM-domain-binding protein family of transcriptional coregulators that have been shown to participate directly in both stimulatory and repressive transcriptional events in other systems by binding to a diversity of DNA-binding proteins (van Meyel et al., 2003). One speculation is that SEU and ANT mediate the action of an unidentified regulator of CRC expression that is differentially localized or active along the apical/basal axis of the gynoecium. Because a variety of experiments indicate that auxin signaling provides positional information along this axis (Sessions et al., 1997; Nemhauser et al., 2000; Balanza et al., 2006), it is possible that this proposed factor would be auxin dependent in some fashion. The proximity of the CRC internal domain expression sites to the sites of ovule initiation, as well as a reduction in ovule initiation in crc mutants, suggest...
that these internal domains may facilitate ovule initiation (Bowman and Smyth, 1999). The loss of the CRC internal domain expression in the seu ant gynoecia is consistent with these domains being important for ovule development. However, the presence of ovule formation, albeit reduced, in crc null alleles indicates that redundant activities for CRC likely exist. We show that small rays or fingers of YAB1/FIL expression extend into the medial domain and may overlap with the subepidermal foci expressing CRC. Given the sequence similarity between YAB1/FIL and CRC, it is possible that YAB1/FIL provides a partially redundant activity for ovule initiation.

The ectopic expression of CRC within the seu ant mutant indicates that SEU and ANT are required for the repression of CRC in the apical portion of the gynoecium. LUG has previously been shown to function as a negative regulator of CRC in perianth organs and a positive regulator of CRC in the internal gynoecium domains (Bowman and Smyth, 1999). In contrast to the ectopic CRC perianth expression reported for the lug mutant, we did not observe ectopic expression of CRC in the perianth organs of the seu, ant, or seu ant mutants (data not shown). We do not yet know the phenotypic significance of the ectopic CRC expression observed in the apical portions of the seu ant gynoecia. It is possible that this ectopic expression contributes to the apical defects of the seu ant mutant (enhanced loss of stigmatic and style tissues) or that misregulation of CRC at the apex contributes to the loss of ovules in the ovarian portions of the gynoecium. Further genetic analysis will be required to test these possibilities.

Differential Contributions of SEU and LUG to Gynoecial and Floral Development

The seu ant mutants fail to initiate ovule primordia and have reduced growth of other medial-ridge-derived tissues. Still the seu ant defect is less severe than the complete loss of the medial domain reported for the lug ant double (Liu et al., 2000). Thus SEU and ANT may regulate a subset of the genes regulated by LUG and ANT or may be less stringently required for the regulation of these genes. We note that in contrast to the lug ant mutant, the seu ant double mutants seldom display homeotic transformations of perianth organs and we did not observe ectopic expression of AG by in situ hybridization (data not shown). Thus, although SEU and LUG function as a molecular complex, SEU and LUG must make independent contributions to the activity of this complex and may be required to differing extents for the action of this complex during diverse regulatory interactions. The non-equivalence of SEU and LUG is further supported by the strong synergistic enhancement of floral phenotypes reported in the seu lug double mutants (Franks et al., 2002). One molecular explanation for a differential requirement for SEU and LUG is that they may have multiple protein partners and may participate in a number of different complexes that are required for diverse developmental events. A family of SEUSS-LIKE (SLK) genes has been described in Arabidopsis (Franks et al., 2002), and Antirrhinum members of this family have been shown to physically interact with STYLOSA, the Antirrhinum LEU ortholog (Navarro et al., 2004). Thus, the SLK gene family may support SEU-independent activities of LUG in the seu mutant plants.

SEU May Be Required for Functions That Are Redundant with ANT during Ovule Initiation and Medial Domain Development

ANT is expressed throughout the gynoecium in early stages and at highest levels in the adaxial core of the gynoecium before ovule initiation (Elliott et al., 1996). During ovule initiation and early ovule development ANT is strongly expressed in the medial ridge and the developing ovule primordia. Thus, ANT likely provides a key proliferative potential to the ovule primordia early in their development. In ant null allele mutants, however, ovule primordia initiate and continue to develop until the time of integument initiation indicating that ANT activity is not absolutely required for ovule initiation, per se (Elliott et al., 1996; Klucher et al., 1996). The complete loss of ovule initiation in the seu ant double suggests that seu may be required to potentiate the activity of ANT-independent pathways that provide a redundant proliferative or organ initiation activity during early ovule development. There are at least two pathways that might provide these redundant activities: YABBY genes and AINTEGUMENTA-LIKE (AIL) genes.

YABBY Gene Family

The observation that fil ant mutants display a near complete loss of the gynoecial medial domain demonstrates that YAB1/FIL provides an important function
that is partially redundant with ANT (Nole-Wilson and Krizek, 2006). The similar disruptions of medial domain development observed in the seu ant and fil ant double mutants suggest that SEU can potentiate the action of ANT in a manner similar to YAB1/FIL. The fil ant flowers also display a loss of floral meristem identity and floral organ identity that is characterized by reduced expression of APETALA3 and ectopic AG expression. These phenotypes are not observed in the seu ant double mutants (data not shown), highlighting a degree of functional differentiation between SEU and YAB1/FIL. Evidence that SEU and LUG may physically cooperate with YAB1/FIL comes from the analysis of orthologous genes in Antirrhinum majus (Navarro et al., 2004). STYLOSA (STY), the Antirrhinum ortholog of LUG, has been shown to interact physically with members of the Antirrhinum YABBY and SLK gene families suggesting that the orthologous Arabidopsis proteins (SEU, YAB1/FIL, CRC, and LUG) may be part of a multimeric complex important for the development of the medial domain of the gynoecium.

Our report of a synergistic genetic interaction in the seu crc double mutant is the first report to our knowledge that demonstrates functional synergy between seu and a member of the YABBY gene family. Although our in situ hybridization results indicate that SEU and ANT work together to promote the expression of CRC within the internal expression domains, the genetic analysis of the seu crc double mutant suggests that SEU and CRC also function in partially redundant, parallel pathways with respect to carpel fusion and stigmatic tissue development.

AIL Gene Family

ANT is a member of a family of transcriptional regulators, the AIL gene family (Nole-Wilson et al., 2005). The expression of several members of the AIL family within the gynoecium overlaps with that of ANT suggesting that these genes may provide some functional redundancy. Interestingly, ail6 ant double mutants display a severe disruption of medial domain development that in some ways resembles that of the fil ant, lug ant, and seu ant double mutants (B. Krizek, unpublished data). One possibility is that AIL6 provides functional redundancy in the ant mutant, where SEU is functional. However, in the seu ant mutant, AIL6 activity may be compromised if its ability to function requires SEU.

A Model for Development of the Gynoecium Medial Domain

We propose a model for the action of SEU, ANT, and LUG during the development of the gynoecial medial domain (Fig. 8). We speculate that the SEU/LUG coregulator complex physically interacts with YAB1 and/or ANT. This multimeric complex can: (1) potentiate the expression of the adaxial fate determinants (PHB and REV) in the adaxial core of stages 6 to 8 developing carpel; (2) support cellular proliferation within the medial ridge and the initiating ovule primordia through a PHB-independent pathway; and (3) engender a position-dependent regulation of CRC expression, supporting CRC expression in the internal expression domains of the gynoecium and repressing CRC expression at the gynoecial apex. These three functions support medial domain development and ovule initiation in wild-type gynoecia. Additional members of the SLK (Franks et al., 2002; Navarro et al., 2004), AIL (Nole-Wilson et al., 2005), YABBY (Siegfried et al., 1999), and LUG/LEUNIG-HOMOLOGIE (Conner and Liu, 2000) gene families are likely to provide molecular redundancy by also participating in this multicomponent molecular complex. Mutant combinations that disrupt at least two of these protein subunits can result in a dramatic loss of the gynoecium medial domain (e.g. lug ant, fil ant, and ail6 ant; Liu et al., 2000; Nole-Wilson and Krizek, 2006; B. Krizek, unpublished data), a loss of ovules from the medial domain (e.g. seu ant; this study), or a near complete deletion of the entire gynoecium (e.g. seu lug; Franks et al., 2002).

MATERIALS AND METHODS

In Situ Hybridization

In situ hybridizations were carried out as previously reported (Franks et al., 2002). A detailed in situ hybridization protocol is available at http://www4.ncsu.edu/~rgfranks/protocols.html. In situ hybridization probes were in vitro transcribed from the following plasmids: ANT, p3delu14; CRC, pCRII_CRCc1; PHB, pPHB-AS (gift of J. Bowman, Monash University, Victoria, Australia); REV, pCRII_REVc4; YAB1/FIL, pY1-Y (gift of J. Bowman).

Microscopy

SEM was carried out as previously reported (Franks et al., 2006) except that the images were collected on a JEOL 5900LV. Chloral hydrate clearing (Berleth and Jurgens, 1993) and Alcian blue staining (Sessions and Zambryski, 1995) were performed as described. In situ, Nomarski, and Alcian-blue-stained samples were imaged on an Axioskop2 microscope (Zeiss) and captured with a MicroPublisher 5.0 RTV digital camera and QCapture software (QImaging). Stereoscope images were collected with the same camera on a MX12.5 stereoscope (Leica). Confocal images were collected on a IMBE inverted confocal microscope (Leica) at standard GFP settings.

Tissue Dissection, RNA Extraction, and qRT-PCR

Whole inflorescences were collected in 100% ice cold ethanol, fixed overnight at 4°C, and then dissected under a MX12.5 stereomicroscope (Leica) while submerged in 100% ethanol. Tissue was stored in 100% ethanol at 4°C or −20°C for up to 1 week while appropriate development stages were dissected. Ethanol was removed and tissue frozen in liquid nitrogen and then ground. RNA extraction was performed using TRI reagent (Molecular Research Center) according to manufacturer’s instructions. The cDNA synthesis was performed using the SuperScript first-strand synthesis system for qRT-PCR (Invitrogen) with a oligo(dT)-primer, according to the manufacturer’s instructions. The qRT-PCR was performed on an ABI 7900 using the Quantitect SYBR Green PCR kit (QIAGEN). In a single PCR run, all samples were done in triplicate, averaged, and normalized using the levels of ADENOSINE PHOSPHORYL TRANSFERASE gene (APT; At1g27450); APT expression levels of the parental ecotype (Col) were set to a relative level of 1.0. Expression levels of PHB and REV were displayed as a fraction of the wild-type (Col) value. Dissociation curve analysis was performed at the end of each run to
ensure the specificity of each reaction. Figure 3, U and V, shows the average relative expression of six PCR runs (three from each of two biological replicates). A Student’s t test for significance was performed on these six normalized averages. Primers used for qRT-PCR analysis are as follows: for PHB, RTPHB-1 and RTPHB-2; for REV, RTREV-1 and RTREV-2. Sequences of these primers are reported in the supplemental data.

Genetic Analysis and Plant Growth

All of the alleles used in this study have been previously described: ant-1 and ant-3 (Kucher et al., 1996) were backcrossed into Col-0 three and seven times, respectively, before our genetics analysis; ant-9 (Elliott et al., 1996); crc-1 (Alvarez and Smyth, 1999); phb-1d (McConnell and Barton, 1998); seu-1 (Franks et al., 2002); seu-3 (Pfluger and Zambryski, 2004). All genotypes were confirmed by PCR-based markers. Details for these markers are provided in the supplemental data. Plants were grown in growth chambers at 22°C to 24°C C with a 16-h/8-h photoperiod under fluorescent light at 80 to 150 μmol m−2 s−1.

Generation of seu ant 35S:PHB Plants

Seeds containing the 35S:PHB construct in a phb-12 pho-11 cnu-2 ath6-12 quadruple mutant background were obtained from Dr. Steven Clark (Prigge et al., 2005). 35S:PHB phb-12 pho-11 cnu-2 ath6-12 plants were crossed to seu-3. F1 progeny were then crossed to ant-1. F1 progeny segregating both the seu-3 and ant-1 alleles were verified by PCR genotyping. This and subsequent generations were used for phenotype analysis. At each step, plants containing the 35S:PHB transgene were identified by selection on plates containing Basta prior to growth in soil. Loss of the original quadruple mutant background was also verified by PCR genotyping as previously described (Prigge et al., 2005).

Generation of phb-1d/+ seu ant 3-ant-1 Plants

phb-1d/+ (heterozygous) seeds were obtained from the Arabidopsis Biological Resource Center (stock CS7361) and grown at 16°C. phb-1d/+ plants were crossed to ant-1. F1 progeny with a phb-1d/+ ant-1/+ genotype were then crossed to seu-3, phb-1d/+ seu-3 ant-1 plants from the F2 and subsequent generations were used for phenotype analysis. All genotypes were confirmed by PCR-based markers. Details for these markers are provided in the supplemental data.

pSEU::SEU:GFP and pSEU::GFP:SEU Constructs

For details of the construction of pSEU::SEU:GFP and pSEU::GFP:SEU constructs, see the supplemental data. Three independent transformant lines for both pSEU::SEU:GFP and pSEU::GFP:SEU were obtained by the simplified Agrobacterium floral dip method (Clough and Bent, 1998). All six of these lines rescued all of the aboveground seu-1 mutant phenotypes (data not shown). Expression from these six transformant lines was consistent between the individual lines. Transformant plants were examined in the T2 and T3 generations.

Supplemental Data

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

Supplemental Figure S1. Details of construction of pSEU::SEU:GFP and pSEU::GFP:SEU.

Supplemental Figure S2. Genotyping for mutant alleles used in this study.

Supplemental Table S1. Oligonucleotides used in this study.

ACKNOWLEDGMENTS

We thank Z. Liu and R. Fischer for support of this project from its inception; J. Pfluger, G. Drews, and C. Grossinger for technical advice; M. Prigge, S. Clark, and J. Bowman for sharing reagents and seeds; B. Krizek for sharing unpublished results; Z. Liu, J. Reed, J. Mahaffey, and anonymous reviewers for helpful comments on the manuscript; the Arabidopsis Biological Resource Center for distribution of seeds; and the North Carolina State University Center for Electron Microscopy and Cellular and Molecular Imaging Facility.

Received December 11, 2007; accepted January 2, 2008; published January 9, 2008.

LITERATURE CITED


Nole-Wilson S, Trantby TL, Krizek BA (2005) AINTEGUMENTA-like (AIL) genes are expressed in young tissues and may specify meristematic or division-competent states. Plant Mol Biol 57: 613–628

Gynoecium Patterning and Ovule Initiation