RABBIT EARS is a second-whorl repressor of AGAMOUS that maintains spatial boundaries in Arabidopsis flowers

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Summary
The RABBIT EARS (RBE) gene has been identified as a regulator of petal development in Arabidopsis thaliana. We find that second-whorl petals in rbe mutants can be replaced with staminoid organs, stamens or filaments and that some rbe flowers have increased numbers of sepals and exhibit fusion of sepals. We show that these rbe defects are due to AGAMOUS (AG) misexpression in the second whorl. Consistent with its role in maintaining the spatial boundary of AG expression, rbe enhanced the second-whorl defects present in ap2-1, lug-1 and clf-2 mutants. In the development of second-whorl organs, RBE acts in the same pathway and downstream of UNUSUAL FLORAL ORGANS (UFO). Enhanced first-whorl organ fusion in ap2-2 rbe-3, ant-4 rbe-3 and cuc2-1 rbe-3 double mutants supports an additional role for RBE in organ separation. RBE thus acts to maintain two different types of spatial boundaries in young flowers: boundaries between organ primordia within a whorl and boundaries of homeotic gene expression between whorls.

Keywords: AGAMOUS, boundaries, cell division, floral patterning, UNUSUAL FLORAL ORGANS (UFO).

Introduction
Arabidopsis thaliana flowers consist of four whorls of organs arranged in a characteristic pattern. Four first-whorl sepals arise in a cruciform pattern, with four second-whorl petals arising between the sepals. The third whorl consists of six stamens: four tall medial stamens and two short lateral stamens. Two congenitally fused carpels make up the inner fourth-whorl gynecium. Organ primordia acquire their distinct identities through the region-specific activities of the ABC class genes (reviewed in Jack, 2004). Sepal identity is specified by the class A genes APETALA1 (AP1) and APETALA2 (AP2). Petal identity is specified by the combined activity of the class A genes and the class B genes APETALA3 (AP3) and PISTILLATA (PI). Stamens are specified by the combined activity of the class B genes and the class C gene AGAMOUS (AG). Carpel identity is conferred by AG activity. The SEPALLATA genes act together with the A, B and C class genes to specify petal, stamen and carpel identities in the flower (Ditta et al., 2004; Pelaz et al., 2000).

In addition to specifying the identity of second-whorl organs, AP1 and AP2 are also required for the initiation of these organs. In ap1 and ap2 mutants, second-whorl organ primordia are not initiated. Because organ initiation is at least partly restored in ap2 ag and ap1 ag double mutants, AG is responsible for the absence of these organs and has been proposed to act as a suppressor of growth in the second whorl (Bowman et al., 1991b, 1993). AG is misexpressed in the outer two floral whorls of ap2, resulting in the conversion of first-whorl sepals into carpels in addition to the absence of second-whorl organ primordia (Drews et al., 1991). Besides AP2, other genes act to repress AG expression in the first and second whorls including LEUNIG (LUG), SEUSS (SEU), CURLY LEAF (CLF), STERILE APETALA (SAP), BELLRINGER (BLR) and AINTEGUMENTA (ANT; Bao et al., 2004; Byzova et al., 1999; Franks et al., 2002; Goodrich et al., 1997; Krizek et al., 2000; Liu and Meyerowitz, 1995; Liu et al., 2000).

Other genes that promote the initiation and development of second-whorl organ primordia include UNUSUAL FLORAL ORGANS (UFO), PETAL LOSS (PTL), RABBIT EARS (RBE) and ROXY1. One class of ufo alleles exhibit an almost
consistent with their roles in the establishment of boundaries between sepals and between stamens can occur in cuc1 cuc2 double mutants (Ishida et al., 2000; Takada et al., 2001). Recently, PTL has been shown to repress growth between sepal primordia, thus contributing to the formation of boundaries between these organs (Brewer et al., 2004). Sepals are sometimes fused in ptl mutants and more often fused in ptl cuc1 and ptl cuc2 double mutants (Brewer et al., 2004).

our work described here reveals that RBE patterns young flowers by maintaining two different types of boundaries. RBE promotes second-whorl development by maintaining a boundary between the second and third whorls of the flower that restricts AG expression to whorls three and four. Although the initial boundary of AG expression may be established independently of RBE, the continued existence of this boundary requires RBE. Our data also suggest that RBE acts to maintain a second type of boundary, one that separates individual sepal primordia within the first whorl. Molecular and genetic evidence suggests that UFO and RBE function in the same pathway to promote the development of second-whorl organs and that UFO is required for normal levels of RBE expression. In addition, we describe the expression pattern of RBE during the development of the ovule and show that RBE also acts to regulate growth of the outer integument.

results

identification of a new rbe allele

We identified a mutant displaying loss of petal identity and reduced fertility that is allelic to rabbit ears (rbe; Takeda et al., 2003). This allele, rbe-3, contains a single nucleotide change (C to T) within RBE that converts Q106 to a stop codon (Figure 1a) and fails to complement the rbe-2 T-DNA insertion allele. rbe-1 and rbe-2 had been described previously as having defects in development of the second whorl of the flower. Second-whorl petals were often missing, reduced in size, altered in shape (described as spoon-shaped) or replaced by filaments (Takeda et al., 2003).

rbe-3 mutants have disrupted development of first-whorl organs

We noticed several phenotypes in rbe-3 that had not been described previously for the other two alleles. rbe-3 flowers sometimes produce more than four sepals (Figure 1b–d) and exhibit fusion between adjacent sepals (Figures 1d and 2a). More than four sepals were observed in approximately 7% of rbe-3 flowers, while some degree of sepal fusion was observed in 35% of flowers (corresponding to 10% of all sepals, Table 1). In rbe-3 flowers with more than four sepals, the ‘extra’ sepal was thinner than the remaining four sepals (Figure 1c). In some cases, one of the adjacent sepals was also somewhat reduced in width. Sepal fusion can occur in the absence of more than four sepals. Sepal fusion was observed most often at the base of these organs (Figure 2a) and more rarely along their entire length (Figure 1d). We measured the width of mature sepals to determine whether sepal fusion might be due to wider organs. rbe-3 sepals were 12% wider than Landsberg erecta (Ler) sepals at maturity (Table 1).

Examination of rbe-3 flowers by scanning electron microscopy (SEM) suggests that sepal fusion in rbe-3 mutants is due to altered patterns of growth within sepal primordia after initiation. During stage 3 of flower development, four

complete loss of second-whorl organs (Durfee et al., 2003). Since UFO encodes an F-box protein that is a component of an SCF complex, it has been proposed that UFO may target for degradation a repressor of whorl-two cell proliferation (Durfee et al., 2003; Samach et al., 1999). In ptl mutants, second-whorl petals can be absent, tubular, staminoid or filamentous in appearance and petal orientation is sometimes altered (Griffith et al., 1999). PTL encodes a trihelix transcription factor that is expressed in four domains corresponding to regions between initiating sepal primordia (Brewer et al., 2004).

rbe mutants have been described previously as having alterations in petal morphology, reduced numbers of petals and conversion of petals to filaments (Takeda et al., 2003). RBE encodes a SUPERMAN (SUP)-like zinc-finger protein that is expressed in second-whorl cells from floral stages 3–6 (Takeda et al., 2003). Recently described roxy1 mutants show reductions in petal number and alterations in petal morphology similar to ptl and rbe mutants (Xing et al., 2005). ROXY1 encodes a glutaredoxin that may post-translationally regulate proteins involved in petal development. UFO has been proposed to function downstream of AP1 in a whorl-two proliferation (Durfee et al., 2003) while RBE has been proposed to function downstream of both AP1 and PTL (Takeda et al., 2003).

Several genes involved in maintaining boundaries within developing flowers have been identified. SUP has been proposed to maintain a boundary between the third and fourth whorls of the flower (Sakai et al., 1995). Mutations in SUP result in misexpression of the class B gene AP3 in the center of the flower and the formation of additional stamens at the expense of fourth-whorl carpels (Bowman et al., 1992). SUP may prevent expansion of AP3 expression into the fourth whorl by regulating the balance of cell division in the third and fourth whorls (Sakai et al., 1995).

The development of discrete organ primordia in the first and third whorls of Arabidopsis flowers requires the activity of the CUP-SHAPED COTYLEDON (CUC) genes. Fusion between sepals and between stamens can occur in cuc1 and cuc2 single mutants but is more common and more dramatic in cuc1 cuc2 double mutants (Aida et al., 1997). Consistent with their roles in the establishment of boundaries, CUC1 and CUC2 are expressed between floral whorls and between individual organ primordia within a whorl (Ishida et al., 2000; Takada et al., 2001). Recently, PTL has been shown to repress growth between sepal primordia, thus contributing to the formation of boundaries between these organs (Brewer et al., 2004). Sepals are sometimes fused in ptl mutants and more often fused in ptl cuc1 and ptl cuc2 double mutants (Brewer et al., 2004).

Our work described here reveals that RBE patterns young flowers by maintaining two different types of boundaries. RBE promotes second-whorl development by maintaining a boundary between the second and third whorls of the flower
sepal primordia are initiated in normal positions in rbe-3 mutants (Figure 2b,c). However, early in stage 4 some rbe-3 sepal primordia have undergone extra growth along their margins and appear wider than normal (Figure 2d,e). The tips of these primordia were sometimes not located in the middle of the primordia and the two sides of the primordia were not symmetrical (Figure 2e). Continued uneven growth of these primordia became more pronounced during development. By stage 5, an organ that had presumably initiated as a single primordium now appeared as two fused primordia (Figure 2f) or two closely spaced and partially fused primordia (Figure 2g).

Petals can be replaced by staminoid organs and filaments in rbe-3

In rbe-3 flowers, second-whorl petals are often reduced in size or altered in appearance. Petals with staminoid characteristics, stamens, filaments or the absence of organs can all be found in the second whorl of rbe-3 mutants (Table 2; Figures 1e–g and 2h–k). In early flowers, the majority of second-whorl organs were petals. Sometimes, the petals were reduced in size or had an altered appearance with the margins of the petal blade folded in toward the center or the petal bent over (Figure 1g). Organs were classified as staminoid based on their overall appearance. Scanning electron microscopy of these organs indicated that some were mosaic organs consisting of both petal-like (Pe) and stamen-like (St) cells (Figure 2l) while others had epidermal cells with morphologies intermediate between that of petals and stamens (Figure 2m). The defects in petal development increased acropetally in rbe-3, with older flowers more likely to have filaments in the second whorl (Table 2, Figure 1f) or to lack second-whorl organs. More severe defects (i.e. the replacement of petals by filaments or the absence of organs) were observed in the two positions closest to the inflorescence meristem. This position effect has been described previously for rbe-1 and rbe-2 (Takeda et al., 2003).
Figure 2. Scanning electron micrographs of rbe-3.
(a) rbe-3 flower in which two sepals are fused at their base (arrow).
(b) Stage 3 Ler flower.
(c) Stage 3 rbe-3 flower.
(d) Ler inflorescence.
(e) rbe-3 inflorescence. Arrows point to regions of abnormal growth in stage 4 sepal primordia.
(f) Close-up of a rbe-3 stage 5 flower in which one sepal primordium is split at its tip (arrow).
(g) Close-up of a rbe-3 stage 5 flower with five sepal primordia. The arrow points to a small sepal primordium located close to a medial sepal.
(h) Ler flower in which the sepals have been removed. Second-whorl petals are visible.
(i) rbe-3 flower containing a second-whorl staminoid organ (arrow).
(j) rbe-3 flower with a second-whorl staminoid petal (arrow).
(k) rbe-3 mosaic second-whorl organ.
(l) Close-up of organ in (k) showing stamen-like (St) and petal-like (Pe) cells.
(m) Epidermal cells present on a rbe-3 second-whorl organ.
(n) Stage 9 Ler flower.
(o) Stage 9 rbe-3 flower. The second-whorl organ primordia (arrows) are smaller than those of the wild type.
(p) Stage 9 rbe-3 flower containing a filament (arrow) in the second whorl.
(q) Ler stage 2-V ovules.
(r) rbe-3 stage 2-V ovules.
(s) Mature Ler ovules.
(t) Mature rbe-3 ovules. The outer and inner integuments (oi and ii) do not fully enclose the nucellus.
(u) Ler ovule showing the narrow width of the outer integument (oi) upon completion of growth around the nucellus.
(v) The outer integument (oi) is not tightly appressed to the inner integument (ii) in some rbe-3 ovules (arrow).

Scale bars correspond to 500 µm in (a), (h) and (i), 200 µm in (j) and (k), 100 µm in (n)–(p), (s) and (t), 50 µm in (d)–(g), (l), (r), (u) and (v), 20 µm in (b), (c) and (l) and 10 µm in (m).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sepal width (mm)</th>
<th>Total no. flowers counted</th>
<th>Fraction of total sepal length exhibiting fusion</th>
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<tr>
<td></td>
<td></td>
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<td>0</td>
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<tr>
<td>Ler</td>
<td>0.66 ± 0.069</td>
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<tr>
<td>rbe-3</td>
<td>0.74 ± 0.095</td>
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<tr>
<td>cuc2-1</td>
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<td>10.9</td>
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<td>cuc2-1 rbe-3</td>
<td>0.69 ± 0.076</td>
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Table 1 Sepal width and extent of sepal fusion in Ler, rbe-3, cuc2-1 and cuc2-1 rbe-3 flowers

Table 2  Organs present in Ler and rbe-3 flowers

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<tr>
<th>Whorl</th>
<th>Organ identity</th>
<th>Ler 1–30&lt;sup&gt;a&lt;/sup&gt; (114)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>rbe-3 1–30&lt;sup&gt;a&lt;/sup&gt; (150)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ler 1–10&lt;sup&gt;a&lt;/sup&gt; (50)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>rbe-3 1–10&lt;sup&gt;a&lt;/sup&gt; (50)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ler 11–20&lt;sup&gt;a&lt;/sup&gt; (50)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>rbe-3 11–20&lt;sup&gt;a&lt;/sup&gt; (50)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ler 21–30&lt;sup&gt;a&lt;/sup&gt; (50)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>rbe-3 21–30&lt;sup&gt;a&lt;/sup&gt; (50)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>1</td>
<td>Se</td>
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<td>4.05</td>
<td>4.04</td>
<td>4.02</td>
<td>4.08</td>
<td>4.08</td>
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<tr>
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<td>Pe</td>
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<td>Folded Pe</td>
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<td>Filament</td>
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<td>0.76</td>
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<tr>
<td></td>
<td>Pe/fil</td>
<td>0.13</td>
<td>0.02</td>
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<tr>
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<tr>
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<td>Pe/St</td>
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<tr>
<td>3</td>
<td>Ca</td>
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<td>2.02</td>
<td>2.02</td>
<td>2.00</td>
<td>2.04</td>
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</tbody>
</table>

<sup>a</sup>Position of flowers on the inflorescence.
<sup>b</sup>Total number of flowers counted.

Se, sepal; Pe, petal; St, stamen; Ca, carpel; St/Pe, staminoid petal; Pe/St, petaloid stamen; Pe/fil, petaloid filament.

initiated in rbe-3 mutants, had a normal appearance. However, by stage 9 of flower development differences in the overall size and shape of wild-type and rbe-3 second-whorl organs were apparent (Figure 2n–p). Second-whorl rbe-3 organs were not as wide as wild-type petals (Figure 2o) and in some cases remained radially symmetric rather than expanding laterally and becoming flat (Figure 2p). To investigate whether the defect in second-whorl organ development might be the result of smaller floral meristems, we measured the diameter of stage 3 floral meristems in Ler and rbe-3 flowers. No difference in floral meristem width was observed (35.2 ± 2.81 μm in Ler versus 35.3 ± 4.85 μm in rbe-3).

Because several rbe-3 phenotypes (extra sepals, fusion of first-whorl sepals and replacement of petals by staminoid organs) had not been reported for rbe-1 or rbe-2 (Takeda et al., 2003), we examined rbe-2 to investigate whether our observations were allele-specific. We found first- and second-whorl defects in the rbe-2 allele similar to those described here for rbe-3, indicating that our observations are not particular to the rbe-3 allele.

rbe-3 mutants show defects in integument growth

To better define the reduced fertility of rbe-3 mutants, we examined ovule development by SEM. Ovule development was normal up to stage 2-V (Schneitz et al., 1995) with both integuments initiating from the chalazal region and growing toward the nucellus (Figure 2q,r). However, later growth of the outer integument in rbe-3 mutants is abnormal with premature cessation of growth. The extent of growth of the outer integument in rbe-3 is somewhat variable, but in most cases the outer integument does not grow to surround the nucellus (Figure 2s,t). In addition, the outer integument is not tightly appressed to the inner integument (Figure 2u,v).

RBE is expressed in petals and the integuments of ovules

Previously, RBE expression has been described in floral organs (Takeda et al., 2003). RBE mRNA was detected in four distinct domains during stages 3–6 of flower development. During stages 3 and 4, the RBE expression domain corresponded to cells that will give rise to petal primordia. RBE was expressed in petal primordia during stage 6 but not during later stages of petal development. We have examined RBE expression in both floral organs and ovules. We found a similar pattern of expression in young flowers (Figure 3b,d) as described previously. However, we also detected RBE expression in late stage 2 flowers (Figure 3a). In some stage 3 flowers, RBE mRNA was detected in a cup-shaped domain (Figure 3c). In ovules, RBE expression was detected in the center of elongated primordia at the time of integument initiation (Figure 3e). RBE mRNA was initially detected in both the inner and outer integument (Figure 3e,f). As the integuments grow around the nucellus, RBE expression is maintained in the inner integument but not the outer integument (Figure 3g,h). Prior to its disappearance in the outer integument, RBE becomes confined to the adaxial side of the outer integument (Figure 3g).

AG activity is required for the first- and second-whorl defects in rbe-3 flowers

Because rbe second-whorl organs are sometimes converted to staminoid petals or stamens, we examined the expression pattern of the class C gene AG in rbe-3 flowers. AG expression was initiated normally in the center of stage 3 rbe-3 floral meristems (Figure 4a,b). However, misexpression of AG in some rbe-3 second-whorl organs was apparent at stage 6 (Figure 4c,d) and continued to stage 12 of flower development (Figure 4e–g). In some cases, AG mRNA was detected throughout the second-whorl organ (Figure 4d) while in other cases AG mRNA was predominantly localized to the adaxial epidermal layer of the second-whorl organ (Figure 4f,g). AG expression was detected in second-whorl organs that appeared to develop as filaments (Figure 4g) as well as those that developed into staminoid petals (Figure 4f). We examined AG expression in ovules of rbe-3 to see whether the defect in growth of the outer integument might result from AG misexpression. AG mRNA was found in a normal pattern in rbe-3 ovules, first appearing throughout ovule primordia and later becoming restricted to the endothelium (Figure 4h,i). This expression pattern matches that described earlier for AG (Bowman et al., 1991a).

To determine whether the rbe second-whorl defect was due entirely to ectopic AG activity, we made an ag-1 rbe-3
Figure 3. *RBE* expression in *Ler* flowers and ovules.
(a) *RBE* expression in a late stage two flower.
(b, c) *RBE* expression in stage 3 flowers.
(d) *RBE* expression in a stage 4 and stage 6 flower.
(e) *RBE* expression in the chalazal region of young ovule primordia (op). Arrows indicate *RBE* expression in the inner (black) and outer (white) integuments that have just initiated from the chalazal region in one of these ovule primordia. The base of the ovule primordia are indicated by op.
(f) *RBE* expression in the inner (black arrow) and outer (white arrow) integuments of ovule primordia.
(g) *RBE* is expressed in the inner integument (black arrows) and on the adaxial side of the outer integument (white arrows) as the integuments grow around the nucellus (stage 2-V).
(h) During stage 2-V, *RBE* expression becomes restricted to the inner integument. Black arrows point to the inner integument of two ovule primordia oriented with the future micropylar end facing forward.
Scale bars correspond to 50 µm in (a)–(h).

Figure 4. *AG* expression in *rbe-3*.
(a) *AG* is expressed in the center of a *Ler* stage 3 floral meristem.
(b) *AG* is expressed in the center of a *rbe-3* stage 3 floral meristem.
(c) *AG* mRNA is detected in the stamen and carpel primordia of a stage 6 flower, but not in the second-whorl petal primordia (arrow).
(d) *AG* mRNA is present in a *rbe-3* second-whorl organ primordia (arrow).
(e) Transverse section of a stage 12 *Ler* flower showing *AG* expression in the stamens but not the petal.
(f) Transverse section of a stage 12 *rbe-3* flower in which *AG* is expressed in the adaxial epidermis of the second-whorl organ (arrow).
(g) Transverse section showing *AG* mRNA in the epidermal layers of two second-whorl organ primordia (arrows).
(h) In mature *Ler* ovules, *AG* mRNA is restricted to the endothelium (black arrow). The white arrow points to the endothelium of a second ovule that was sectioned at an oblique angle.
(i) *AG* mRNA is restricted to the endothelium in *rbe-3* mature ovules (black arrow).
Scale bars correspond to 50 µm in (a)–(i).
double mutant. The ag-1 rbe-3 double mutant has the same phenotype as ag-1 single mutants with completely normal petals developing in the second whorl (Figure 5a). In addition, sepal number was normal in these flowers and no cases of sepal fusion were observed. The epistasis of ag-1 to rbe-3 indicates that AG function is required for the rbe-3 first- and second-whorl defects. Thus, RBE is not required for petal development per se but rather to prevent AG misexpression in the second whorl.

rbe-3 enhances the petal to stamen transformations of ap2-1, lug-1 and clf-2 mutants

To further investigate the role of RBE in regulation of AG, we constructed double mutants with genes known to play roles in AG repression. Because strong ap2 mutants do not produce second-whorl organs, we investigated the effect of rbe-3 in the weak ap2-1 background. ap2-1 mutants have leaf-like organs in the first whorl and staminoid petals in the second whorl (Figure 5b; Bowman et al., 1989). The ap2-1 rbe-3 double mutant showed an additive phenotype in the first and fourth whorls. Second-whorl organs of the double mutant were more completely converted to stamens or were missing entirely (Figures 5b and 6a). Because it was sometimes difficult to distinguish second- and third-whorl organs, we counted the total number of stamens in each flower. The average number of stamens in ap2-1 rbe-3 flowers was 6.8 compared with 5.8 per ap2-1 flower. Examination of young ap2-1 rbe-3 flowers by SEM indicated that organ number was not increased in the third whorl. Thus, the increased total stamen number in ap2-1 rbe-3 flowers was due to the presence of stamens in the second whorl. We also observed fusion between stamens (either between second- and third-whorl stamens or between third-whorl stamens) in approximately 24% of ap2-1 rbe-3 flowers (Figure 6b) compared with only 3% in rbe-3 flowers.

We investigated the effect of combining rbe-3 with mutations in LUG, another repressor of AG. lug-1 flowers have first-whorl sepals that are sometimes petaloid or staminoid, a reduced number of second-whorl petals and carpels that are unfused at their tips (Figure 5c; Liu and Meyerowitz, 1995). The first, third and fourth whorls of lug-1 rbe-3 double mutants exhibit an additive phenotype. The number of second-whorl organs was reduced in lug-1 rbe-3 double mutants (from 2.66 per flower in lug-1 to 0.36 in lug-1 rbe-3 and these remaining organs were primarily staminoid (Figures 5c and 6c). Thus, rbe-3 enhanced the second-whorl phenotype of lug-1 mutants. The double mutant rarely produced any seeds, suggesting a more severe defect in female fertility in the double mutant compared with either single mutant. However, no enhancement of the outer integument growth defect was observed in lug-1 rbe-3 flowers (Figure 6d).

We also found genetic interactions between rbe-3 and clf-2 (Goodrich et al., 1997). Under our growth conditions, clf-2 mutants exhibited curling of both rosette and cauline leaves and reduction in petal size in the second whorl (Figure 5d). clf-2 rbe-3 double mutants exhibited leaf curling similar to
that in clf-2. Both clf-2 and clf-2 rbe-3 produced an average of 4.0 second-whorl organs per flower. In clf-2 mutants these were almost exclusively small petals (3.97 per flower) while the average number of small petals in clf-2 rbe-3 was 1.37. Second-whorl petals were most often replaced by spoon-shaped petals (0.29 per flower), staminoid petals (0.91 per flower), petaloid stamens (0.78 per flower) or stamens (0.48 per flower; Figure 5d). Thus, rbe-3 enhanced the transformation of clf-2 second-whorl organs to stamens and stamen-like organs.

We examined the expression of AG in ap2-1 rbe-3 and clf-2 rbe-3 plants to determine whether the enhancement of the second-whorl phenotypes in these double mutants resulted from AG misexpression. High levels of AG mRNA were detected not only in the third and fourth whorls of these flowers but also ectopically in the second whorl, confirming our hypothesis (Figure 7a,b). In contrast, the class B gene AP3 was correctly expressed in second- and third-whorl organs in ap2-1 rbe-3 and clf-2 rbe-3 double mutant flowers (Figure 7c,d). This normal pattern of AP3 expression parallels that observed previously in rbe-1 single mutants (Takeda et al., 2003).

ufo is epistatic to rbe in development of the second whorl

The UFO gene has been shown to promote petal initiation and development by inhibiting AG activity in the second whorl (Durfee et al., 2003). To determine whether RBE might function in a common pathway with UFO, we crossed rbe-3 to the weak ufo-11 and strong ufo-2 alleles. ufo-11 is a weak allele specifically affecting petal development (Durfee et al., 2003). Petals are usually missing in ufo-11 flowers or are sometimes replaced by filaments or petal/stamen mosaic organs. ufo-11 flowers have fewer second-whorl organs compared with rbe-3 mutants and also sometimes produce mosaic petaloid stamens in the third whorl. ufo-11 rbe-3 double-mutant flowers have a phenotype that is similar to ufo-11 flowers (Figure 5e). The total number of second-whorl organs is approximately the same (0.69 second-whorl organs in ufo-11 flowers compared with 0.58 organs in
ufo-11 rbe-3). The numbers of petals (0.048 in ufo-11 versus 0.055 in ufo-11 rbe-3) and mosaic organs (0.19 in ufo-11 versus 0.22 in ufo-11 rbe-3) is the same in both the single and double mutant. However, we did observe a significant increase in the number of stamens (0.014 in ufo-11 versus 0.132 in ufo-11 rbe-3) and a slight decrease in the number of filaments (0.43 in ufo-11 versus 0.18 in ufo-11 rbe-3) in the double mutant. Thus, rbe-3 seems to slightly enhance the phenotype of ufo-11.

ufo-2 mutants show homeotic transformations of floral organ identity, most often in the second and third whorls (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Second-whorl petals are often replaced with sepals and third-whorl stamens are often replaced by carpels (Figure 5f). In addition, filaments and mosaic organs are commonly found in these whorls. The ufo-2 rbe-3 double mutant has the same phenotype as the ufo-2 single mutant (Figure 5f). There is no increase in the number of filaments and/or staminoid organs in the double mutant as compared with ufo-2. These genetic results suggest that UFO and RBE work in a single pathway to promote petal initiation and development in the second whorl. The similarity of the RBE and UFO expression patterns in stage 3 flowers is consistent with this model (Lee et al., 1997).

To determine the order of gene actions (i.e. whether UFO acts upstream of RBE or vice versa), we examined RBE expression in ufo mutants and UFO expression in rbe mutants. RBE expression is dramatically reduced in ufo-11 mutants (Figure 8a–d). In most ufo-11 flowers, no RBE mRNA could be detected. UFO expression is normal in rbe-3 mutants as compared with wild-type flowers (Figure 8e–h). Both the level and pattern of UFO expression are the same in rbe-3 and Ler inflorescences. Our data are consistent with UFO acting upstream of RBE and being required for normal levels of RBE expression.

Although the second-whorl petal loss phenotype is more severe in weak ufo mutants than in rbe mutants, AG mRNA was not detected in the second whorl of ufo flowers (Durfee et al., 2003). It was therefore suggested that AG does not act autonomously in second whorl cells of weak ufo alleles (Durfee et al., 2003). These data argue against RBE and UFO acting in a single common pathway. However, it is also possible that AG misexpression in weak ufo mutants was not detected due to technical challenges. Because petal primordia arise from just two cells in a flower primordium (Bossinger and Smyth, 1996), very early misexpression of AG would be difficult to observe by in situ hybridization. Furthermore, the almost complete loss of second-whorl organs in most ufo flowers would probably prevent detection of later AG misexpression. Because a few second-whorl organs are produced in the earliest-arising flowers of weak ufo alleles, we examined AG expression in ufo-14 flowers by in situ hybridization. We observed AG expression in some second-whorl organs of ufo-14 flowers (Figure 8i–k). Our results are consistent with the defects in weak ufo alleles being due to AG misexpression in the second whorl. The less severe petal loss phenotype in rbe as compared with the weak ufo alleles might result from UFO regulating other AG repressors in addition to RBE.
Figure 8. RBE, UFO, and AG expression in wild type, ufo, and rbe mutants.

(a–d) RBE expression in Columbia (Col) and ufo-11.

(a) RBE expression in a stage 3 Col flower.
(b) No RBE mRNA was detected in this stage 3 ufo-11 flower.
(c) RBE expression in a stage 5 Col flower.
(d) No RBE mRNA was detected in this stage 5 ufo-11 flower.

(e–h) UFO expression in Ler and rbe-3 inflorescences.

(e) In Ler plants, UFO mRNA is present in the inflorescence meristem (IM) and in a cup-shaped domain in stage 3 flowers.
(f) In rbe-3 plants, UFO is expressed in the inflorescence meristem (IM) and in a cup-shaped domain in stage 3 flowers.
(g) UFO is expressed between the sepals (Se) and petals in this stage 7 Ler flower. The arrow points to a second-whorl petal primordium.
(h) UFO is expressed between the sepals and second-whorl organs in this stage 7 rbe-3 flower. The arrow points to a second-whorl organ primordium.

(i–k) AG expression in ufo-14.

(i) AG mRNA is detected in a second-whorl organ of this ufo-14 flower (indicated by an arrow).
(j) Close-up of the second-whorl organ shown in (i).
(k) AG mRNA is detected in a second-whorl staminoid petal of this ufo-14 flower (arrow).

Scale bars correspond to 50 μm in (a)–(k).
ap2-2 rbe-3 and ant-4 rbe-3 double mutants show enhanced first-whorl organ fusion

Strong ap2-2 alleles have carpels or staminoid carpels in medial first-whorl positions and leaf-like organs in the lateral positions (Figure 5g; Bowman et al., 1991b). The first-whorl staminoid carpels are mosaic organs consisting of a central region of carpel tissue fused on one or both sides with stamen tissue (Figure 6e). Second-whorl organs are missing in ap2-2 flowers and there are a reduced number of third-whorl stamens. In ap2-2 rbe-3 double mutants, first-whorl carpels and staminoid carpels were often wider than in ap2-2 (Figure 6f). These organs had the appearance of several fused organs but were only connected to the base of the flower in the central part of the organ and were in a position normally occupied by a single organ. In addition, first-whorl organs were often fused along much of their length (Figures 5g and 6g). All of the first-whorl organs were sometimes fused such that the fourth whorl carpel poked through a ring of tissue (Figure 6h). Cells present along the margin of fusion resembled the epidermal cells normally present on the abaxial replum of wild-type carpels. Thus, the function of RBE in the separation of floral organs does not depend on the identify of the organs in the first whorl. The number of first-whorl organs in the double mutant was slightly increased compared with 3.56 in ap2-2). No second-whorl organs were present in the double mutant and the third and fourth whorls had an additive phenotype.

ant-4 flowers have the normal pattern of organ identity but show reductions in both organ number and size (Figure 5h; Elliott et al., 1996; Klucher et al., 1996). ant-4 rbe-3 double mutants had an increased number of sepal compared with either single mutant (4.42 sepals in ant-4 rbe-3 compared with 3.98 in ant-4). In early arising flowers as many as eight sepals were present. In addition, fusion between sepals was observed in ant-4 rbe-3 mutants (Figures 5h and 6i). Sepals in the double mutant were often fused along their entire length rather than just at their base as usually seen in rbe-3. The initiation pattern of sepal primordia was often disrupted in ant-4 rbe-3 double mutants. One or more of the primordia were sometimes initiated in positions shifted from the typical medial and lateral positions (Figure 6j,k). This resulted in the four primordia not being equally spaced from each other. In some cases, the positions of four sepal primordia were fairly normal but an additional sepal primordium arose in a region between a medial sepal and a lateral sepal (Figure 6i).

ant-4 rbe-3 flowers also show a reduction in the overall number of second-whorl organs (3.8 in rbe-3 compared with 2.5 in ant-4 rbe-3). In addition, fewer filaments (1.2 in rbe-3 compared with 0.7 in ant-4 rbe-3) and staminoid organs (0.5

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in rbe-3 compared with 0.04 in ant-4 rbe-3) were present in the double mutant compared with rbe-3 single mutants. Reduced stamen number in the third whorl of ant-4 mutants was partially rescued by rbe-3. ant-4 mutants have 4.3 stamens per flower whereas ant-4 rbe-3 flowers produce 5.6 stamens per flower.

cuc2-1 rbe-3 double mutants show increased frequency and extent of sepal fusion

To further investigate the role of RBE in organ separation, rbe-3 was crossed to cuc2-1. In the cuc2-1 single mutant, sepals are sometimes fused at their base (Aida et al., 1997; Figure 9a). In rbe-3 cuc2-1 flowers, sepal fusion occurred more often and to a greater extent than in either single mutant (Figure 9b,c; Table 1). The width of cuc2-1 and cuc2-1 rbe-3 sepals is the same at maturity (Table 1), suggesting that the increased amount of fusion in this double mutant is not due to increased organ width. Examination of sepal development in the cuc2-1 rbe-3 double mutant by SEM showed that four distinct sepal primordia are initiated in a normal pattern during stage 3 of flower development as was seen previously for cuc2-1 and rbe-3 single mutants. Later growth of sepal primordia in the cuc2-1 rbe-3 double is uneven, similar to that seen in rbe-3 single mutants. An increased extent of sepal fusion in the cuc2-1 rbe-3 double mutant as compared with cuc2-1 was first observed during stage 6 (Figure 9d-g).

Discussion

RBE is a new repressor of AG expression

Our studies have identified RBE as a new repressor of AG expression in Arabidopsis flowers. The defects observed in the second whorl of rbe mutants (reduced organ number, replacement of petals by filaments and staminoid organs) result from ectopic expression of AG in second-whorl cells. In the absence of AG activity, development of both first- and second-whorl organs is normal in rbe mutants. RBE encodes a potential transcriptional repressor. It contains a single Cys2His2 zinc-finger DNA-binding domain and an ethylene-responsive element binding factor (ERF)-associated amphiphilic repression (EAR)-like domain (Takeda et al., 2003). The related protein SUPERMAN (SUP), which contains a similarly arranged single zinc-finger domain and EAR-like motif, has been shown to possess both DNA-binding and transcriptional repression activities (Dathan et al., 2002; Hiratsu et al., 2002). SUP and RBE are similar in that mutations in either gene disrupt the ability to maintain the boundaries of floral homeotic gene expression.

All but one of the previously identified AG repressors prevent AG transcription in both the first and second whorls of the flower. We have never observed carpelloid features on first-whorl sepals or misexpression of AG in first-whorl organs of rbe mutants, suggesting that RBE acts only within the second whorl to repress AG expression. This is similar to the proposed function of ANT in AG repression (Krizek et al., 2000). The spatially restricted expression pattern of RBE also contrasts with that of other known AG repressors that are transcribed in all four whorls of the flower (Conner and Liu, 2000; Goodrich et al., 1997; Jofuku et al., 1994). The recent demonstration that AP2 is regulated at the translational level by miRNA172 suggests one possible mechanism for restriction of AG repressor activity to the outer whorls of the flower (Aukerman and Sakai, 2003; Chen, 2004). Another possibility is that globally expressed AG repressors require whorl-specific factors like RBE for their activity.

Plant Cys2His2 zinc-finger proteins containing the conserved QALGGH sequence have been shown to bind 5`-AGT-3` sequences. In SUP, the single zinc-finger domain and adjacent basic residues on either side of the zinc finger are sufficient to bind such sequences (Dathan et al., 2002). Basic residues are also present on either side of the RBE zinc finger; those at the carboxy-terminal end of the zinc finger are present at conserved positions with those in SUP. Cis-acting regulatory sequences required for proper spatial expression of AG are located in the 3 kb second intron (Busch et al., 1999; Deyholos and Sieburth, 2000; Sieburth and Meyerowitz, 1997). We examined this intron for the presence of AGT elements and found a total of 102 such sequences on either strand. The expected number of AGT sequences on either strand of a 3 kb piece of DNA is 94. Although the total number of AGT sequences is not significantly greater than the expected number, six of these sequences are present within regions conserved among 29 Brassicaceae species (Hong et al., 2003). Further work will be necessary to determine whether RBE binds directly to AG regulatory sequences.

Pathways regulating petal development

Mutations in UFO, RBE or PTL cause somewhat similar defects in the outer two whorls of the flower. Sepal fusion can occur in the first whorl of ufo-11 (B. Krizek, unpublished data), similar to that observed in rbe-3 and ptl-1 (Griffith et al., 1999). In the second whorl, mutations in any of these three genes results in loss of petals and/or their replacement with filaments or staminoid organs. Our results suggest that RBE and UFO are components of a common pathway that regulates petal initiation and development. RBE transcripts are dramatically reduced in ufo mutants, indicating that UFO is required for RBE expression. Furthermore, AG misexpression in the second whorl occurs in both rbe and weak ufo alleles, and the second-whorl defects in these mutants can be eliminated by the loss of AG activity.

UFO has been shown to function as part of a SCF\textsuperscript{UFO} complex in flowers. Such complexes act as E3 ubiquitin ligating enzymes in the ubiquitin–proteasome protein complex.
degradation pathway. Interestingly, mutations in other components of the SCF<sub>UFO</sub> complex result in carpeloid sepals, sepal fusion and dramatic reductions in petal number (Ni et al., 2004). These phenotypes are present in axr6-2 and ask1/ask1 ask2/ASK2 plants and require AG activity. Thus SCF complexes have been proposed to repress AG function in the outer whorls and to play a role in organ separation by regulating cell division in each whorl (Ni et al., 2004). Our results are consistent with the SCF<sub>UFO</sub> complex mediating proteolysis of a repressor of RBE expression in the second whorl.

Whether PTL is a component of the UFO/RBE pathway or a separate pathway remains unclear. RBE has been proposed to function downstream of PTL in the development of second-whorl organs, as RBE is not expressed in ptil mutants (Takeda et al., 2003). However, AG activity is not responsible for the ptil second-whorl defects as they are still present in ptil ag double mutants (Griffith et al., 1999). This argues against the idea of RBE acting directly downstream of PTL. An alternative possibility is that PTL may act as a growth suppressor in regions between adjacent sepals, and its effect on second-whorl development (and RBE expression) may be an indirect consequence of excessive growth within the inter-sepal domain (Brewer et al., 2004). Further experiments will be required to determine whether PTL and RBE act together in organ formation in the first and/or second whorls. PTL and RBE are expressed in adjacent groups of cells in the first and second whorls. The similarity of the ptil and rbe phenotypes suggest that signaling between these two populations of cells is critical for regulating organ growth during early stages of flower development.

**RBE may be a component of an inter-whorl signaling pathway that regulates growth in the outer two floral whorls**

RBE is only expressed in second-whorl cells (Takeda et al., 2003), yet mutations in this gene alter growth in both the first and second whorls. Decreased growth in the second whorl of rbe mutants appears to be accompanied by increased growth in an adjacent region of the first whorl. We propose that signaling pathways act between adjacent groups of cells in the first and second whorls to regulate cellular proliferation. In our model (Figure 10), UFO and RBE act in one pathway by promoting proliferation of second-whorl cells through repression of AG, which acts as a suppressor of growth when misexpressed in the second whorl (Bowman et al., 1991b, 1993). Proliferating second-whorl cells at the sites of petal initiation generate a signal (S) that moves to adjacent first-whorl cells, activating a first-whorl inhibitor (I) of growth in these regions. Such a pathway results in petal initiation and growth as well as the establishment of boundaries between adjacent sepal primordia. In this model, the amount of second-whorl cell proliferation and thus the amount of S generated is controlled by the activities of UFO and RBE. ROXY1, a glutaredoxin protein (Xing et al., 2005), may also play a role here as a potential post-translational regulator of RBE. In rbe flowers, expression of AG in the second whorl inhibits cell proliferation in the second whorl. This results in reduction or elimination of the growth inhibition signal to the first whorl, leading to increased growth in the inter-sepal zone and consequently sepal–sepall fusion and the occasional formation of extra sepals.

**Experimental procedures**

**Plant growth conditions, mutant isolation and strain construction**

Arabidopsis plants were grown in a 4:1:1 mix of Fafard 4P:perlite:vermiculite at 22°C in continuous light. The rbe-3 allele was
isolated from a mutagenized population of 3SS::PI seeds in the Nossen (No-O) ecotype. The rbe-3 allele was backcrossed four times to Ler prior to phenotypic analyses. All double mutants were created by manual cross-pollination using homozygous mutant strains, except for ag-1. We believe the weak ufo allele used in our genetic studies is ufo-11. Polymerase chain reaction (PCR) genotyping and sequencing of PCR products from this allele indicated that the ufo-12, ufo-13 and ufo-14 molecular lesions are not present. The genotype of ufo-11 rbe-3 double mutants was confirmed using cleaved amplified polymorphic sequences (CAPS) markers for the rbe-3 mutation and crossing of the plants to ufo-11.

**Scanning electron microscopy**

Samples for SEM were fixed, dried, dissected and coated as described previously (Krizek, 1999). Images were collected on a XL30. To measure the widths of stage 3 floral meristems, images were captured at a magnification of 800·, described previously (Krizek, 1999). Images were collected on a XL30. The width of the dome of the floral meristem was measured from the middle of one medial sepal to the middle of the opposite medial sepal. Ten stage 3 floral meristems were measured for Ler and 13 for rbe-3.

**Sepal width measurements**

All sepals were removed from mature flowers corresponding to positions 1–12 on the inflorescences of four individual plants of each genotype. The sepals were laid on their adaxial side and their width measured with an ocular micrometer.

**In situ hybridization**

Inflorescences were fixed, embedded, sectioned, hybridized and washed as described previously (Krizek, 1999). Digoxigenin-labeled RNA probes were synthesized by in vitro transcription using T7 RNA polymerase. The AG antisense probe was made by digestion of pCIT565 (Yanofsky et al., 1990). For the RBE antisense probe, a fragment of RBE corresponding to nucleotides 273–580 was cloned into pCR2.1 and linearized with BamI. The UFO antisense probe was made by digestion of pDW221.1 with Cial (Lee et al., 1997).

**Acknowledgements**

We thank the Salk Institute Genomic Analysis laboratory for providing the Arabidopsis T-DNA insertion mutant, the Arabidopsis Biological Resource Center for seeds of the T-DNA insertion mutant (SALK_037010) and cuc2-1, Detlef Weigel for the UFO in situ plastid pDW221.1 and Patricia Zambrzycki for the weak ufo alleles. This work was supported by a University of South Carolina Research and Productive Scholarship award to BAK and by a United States Department of Agriculture CRIS grant to JCF.

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