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***SEUSS* and *LEUNIG* regulate cell proliferation, vascular development and organ polarity in *Arabidopsis* petals**

Received: 10 February 2006 / Accepted: 7 March 2006
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Abstract Unlike in animals where cell migrations and programmed cell death play key roles in organ shape determination, in plants organ shape is largely a result of coordinated cellular growth (cell divisions and cell elongations). We have investigated the role of the *SEUSS* and *LEUNIG* genes in *Arabidopsis thaliana* (L.) Heynh. petal development to better understand the molecular mechanisms through which cellular growth and organ shape are coordinated in plants. *SEUSS* and *LEUNIG* encode components of a putative transcriptional regulatory complex that controls organ identity specification through the repression of the floral organ identity gene *AGAMOUS*. *SEUSS* and *LEUNIG* also regulate petal shape through *AGAMOUS*-independent mechanisms; however, the molecular and cellular actions of *SEUSS* and *LEUNIG* during petal development are unknown. Here we show that *SEUSS* and *LEUNIG* control blade cell number and vasculature development within the petal. Furthermore, *SEUSS* and *LEUNIG* regulate petal polarity along the adaxial/abaxial axis. We present a model where *SEUSS* and *LEUNIG* are required to potentiate the key polarity genes *PHABULOSA* and *FILAMENTOUS FLOWER/YABBY1* and thus influence cellular growth within the developing petal blade.

Keywords Adaxial · *FILAMENTOUS FLOWER* · *GRAMINIFOLIA* · *PHABULOSA* · *STYLOSA* · *YABBY*

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Abbreviations HD-ZIP: Homeodomain-leucine zipper · SEM: Scanning electron microscopy · s.d.: Standard deviation · *FIL/YABI*: *FILAMENTOUS FLOWER/YABBY1* · *PHB*: *PHABULOSA*

Introduction

Uncovering the mechanisms that control organ shape and size is an important challenge for developmental biologists. The diversity of leaf and floral organ shapes between species and the consistency of the shape within a given species suggest a tight genetic control over organ shape regulation. In plants, the frequency of cell divisions, the orientation of cell division planes, and the extent of cell elongations must be precisely orchestrated to generate an organ of the proper size and shape. Little is known about the underlying genetic network and the mechanisms that coordinate these processes that specify organ shape and size.

In many dicotyledonous plants the lateral organs, particularly leaves and petals, are flattened and blade-like in shape. In leaves, this characteristic shape results from the coordinated growth of cells within the adaxial portion of the developing organ primordium (i.e. the portion that is adjacent to the shoot apex) (Avery 1933; Poethig and Sussex 1985a, b). Cells within the adaxial primordium divide more extensively than those within the abaxial portion (i.e. those further away from the shoot apex) thus generating the laterally extended leaf lamina. Using surgical techniques, Sussex demonstrated that physical connectivity between the shoot apex and the developing leaf primordium was required to generate laminar growth within the leaf (Sussex 1954, 1955). These experiments suggested the existence of a signaling gradient emanating from the shoot apex that was required for adaxial identity and lamina formation. Genetic analysis of leaf development in the *phantastica* mutant in *Antirrhinum majus* further supported the existence of a factor required for adaxial identity and suggested that a juxtaposition between adaxial and abaxial

identities were required for the differential cell growth associated with blade outgrowth (Waites and Hudson 1995).

More recent analyses have identified three major families of transcriptional regulators that are required for the establishment of adaxial/abaxial polarity and for laminar leaf blade growth in *Arabidopsis*: the class III HD-ZIP, YABBY and KANADI protein families (Emery et al. 2003; Eshed et al. 1999, 2001, 2004; Kerstetter et al. 2001; McConnell and Barton 1998; McConnell et al. 2001; Prigge et al. 2005; Sawa et al. 1999; Siegfried et al. 1999; Zhong and Ye 2004). Three members of the class III HD-ZIP genes, *PHABULOSA*, *PHAVULOTA*, and *REVOLUTA*, are expressed most strongly in the adaxial domains of the developing leaf primordium (Eshed et al. 2001; Kerstetter et al. 2001; McConnell et al. 2001; Otsuga et al. 2001; Zhong and Ye 1999). Members of the YABBY and KANADI gene families are expressed in a complementary fashion, being restricted to the abaxial domain (Eshed et al. 2001; Kerstetter et al. 2001; Kumaran et al. 2002; Sawa et al. 1999; Siegfried et al. 1999). Analysis of both gain-of-function and loss-of-function alleles indicates the action of these genes defines adaxial and abaxial leaf fates as well as stimulates leaf laminar growth (Emery et al. 2003; Eshed et al. 1999, 2001; Kerstetter et al. 2001; McConnell and Barton 1998; McConnell et al. 2001; Siegfried et al. 1999; Zhong and Ye 2004).

Although much is known about the organ identity genes that specify petal identity and thus distinguish petal from leaf and other floral organs (Lohmann and Weigel 2002; Zik and Irish 2003), less is known about the molecular mechanisms that generate the characteristic shape of the petal. As the petal is thought to have evolved from a leaf-like precursor (discussed in Honma and Goto 2001; Theissen and Saedler 2001), it is likely that some of the molecular mechanisms that are required for blade outgrowth in the leaf have been recruited to enable the expansion of the petal blade. Consistent with this presumption, some members of the class III HD-ZIP, YABBY and KANADI gene families are expressed in developing petals in an abaxial- or adaxial-specific pattern similar to that seen in the leaf (Otsuga et al. 2001; Sawa et al. 1999; Siegfried et al. 1999). However, little is known about the role of these genes in petal development and the molecular identities of the signals that are responsible for the activation and maintenance of these key polarity determinants in the petal are unknown.

We are studying the action of two transcriptional co-regulators, *SEUSS* (*SEU*) and *LEUNIG* (*LUG*), that mediate proper petal shape as well as organ identity specification in *Arabidopsis*. The *seuss* and *leunig* mutations are highly pleiotropic and display developmental defects in leaves, other floral organs, plant height, control of axillary branching, and root development (Franks et al. 2002; Pfluger and Zambryski 2004). Thus *SEU* and *LUG* function in numerous developmental pathways and likely use functionally different mechanisms to alter

these varied developmental processes. In this manuscript we focus only on the defects observed in the developing petal. *SEUSS* and *LEUNIG* function during petal identity specification by repressing *AGAMOUS* (*AG*) expression (Conner and Liu 2000; Franks et al. 2002; Liu and Meyerowitz 1995). The partial homeotic transformation of petals into stamenoid petals observed in *seu* and *lug* mutants is mediated by ectopic *AG* expression (Franks et al. 2002). *AG* encodes a MADS box-containing transcription factor that promotes stamen and carpel identity (Yanofsky et al. 1990). *LUG* displays protein sequence similarity to both the Tup1/Gro transcriptional co-regulator family as well as to the Ssdp family of transcriptional co-regulators (Conner and Liu 2000). *SEU* is related to the LDB family of transcriptional co-regulators (Franks et al. 2002). Members of these co-regulator families do not appear to possess DNA-binding domains, but are thought to regulate transcription through interactions with DNA-binding transcription factors (Agulnick et al. 1996; Bach et al. 1997; Fisher and Caudy 1998; Flores-Saaib and Courey 2000; Jurata and Gill 1997; Morcillo et al. 1997; van Meyel et al. 2003). The *SEU* and *LUG* proteins physically interact and mediate transcription repression in yeast and in *Arabidopsis* protoplasts when tethered to the DNA through a chimeric DNA binding domain (Sridhar et al. 2004). These experiments suggest a model in which *SEU* and *LUG* form a transcriptional repression complex that mediates *AG* repression in developing sepals and petals for proper organ identity specification (Franks et al. 2002; Liu and Meyerowitz 1995; Sridhar et al. 2004).

In contrast to their role in organ identity specification, the action of *SEU* and *LUG* during petal shape regulation is independent of *AG* activity. While the removal of ectopic *AG* activity in a *seu* or *lug* mutant background restores proper specification of organ identity, it does not restore proper petal shape (Franks et al. 2002; Liu and Meyerowitz 1995). These results argue for a novel *AG*-independent molecular mechanism during petal development. However, the downstream targets of *SEU* and *LUG* regulation in this pathway are presently unknown.

In this paper we examine the *AG*-independent effects of *SEU* and *LUG* on petal development. We find that *SEU* and *LUG* activities are required for the proper development of the petal blade lamina. We show that *SEU* and *LUG* control cell division during petal development and enable the proper patterning of petal blade vasculature. Furthermore, we demonstrate that *SEU* and *LUG* are required for the proper elaboration of petal polarity along the adaxial/abaxial axis. This is supported by altered epidermal cell surface morphology as well as reduced expression of *PHB* and *FIL/YABI*, key regulators of adaxial and abaxial fate, respectively. We present a model in which *SEU* and *LUG* are directly or indirectly required for the expression of *PHB* and *FIL/YABI* and thus regulate cellular proliferation within the developing petal blade.

Materials and methods

Morphometric analysis

All analysis of petal blade area and petal cell size was done in fully mature petals from whorls 2 and 3. To ensure that we were looking at fully mature petals, we first identified flowers that had just undergone the senescence and abscission of their sepals. We then examined the four flowers from the same inflorescence that were just apical to the sepal-less flower (thus representing four younger plastochrons). Area of the petal blade ($N=15$) and the cross-sectional area of individual cells ($N>135$) were estimated from dark field illuminated images of chloral hydrate-cleared petals that were scanned and then analyzed with the ImageJ software (NIH). Whole petal length and width measurements were determined under a Zeiss Stemi SV11 dissecting scope under dark field illumination using a Peak Glass scale 50. Vascular elements were counted in cleared petals under dark field illumination. To determine statistical significance Student's t test was applied; all values reported here as significant returned a P value of <0.001 except comparison of *ag-1* with *seu-1 ag-1* petal width (Fig. 2) where $P=0.017$.

In situ and microscopic analysis

Scanning electron microscopy (SEM) samples were collected, fixed and coated as previously described (Bowman et al. 1989, 1991). Samples were analyzed on an environmental SEM (Electroscan E3 ESEM). Digital images were captured with IAAS software (Electroscan) and adjusted and cropped with Photoshop. Floral tissue for in situ hybridization was fixed as previously described (Drews et al. 1991) except that fixation time was limited to 2 h. The in situ hybridization was carried out according to previously described protocols (Lincoln et al. 1994; Long and Barton 1998) with the following modifications: probes were not hydrolyzed prior to use; and the RNase and acetyl nitrile incubation steps were omitted. The in situ hybridizations were done with control tissue on each slide or as sandwiched pairs of slides to ensure equivalent hybridization efficiency for wild type and mutant tissues.

Plant growth and genetic analysis

Seeds were sown in a mixture of Scotts Metro Mix 200 and Vermiculite (3:1) then imbibed and cold treated at 4–6°C for 3 days in the dark. Plants were then grown in a temperature controlled greenhouse (75–85°F) under 16:8 h day/night regimen. Analysis of plants was carried out at 40 days post germination. The *lug-1*, *seu-1* and *ag-1* alleles were all generated in the Landsberg *erecta* ecotype and have been previously reported (Bowman et al. 1989; Franks et al. 2002; Liu and Meyerowitz 1995), as has the generation of the *seu-1 ag-1*, *ag-1 lug-1* and *seu-1*

ag-1 lug-1 triple mutant strains (Franks et al. 2002). All mutant genotypes were confirmed with PCR-based molecular markers as previously described (Franks et al. 2002).

Results

seu and *lug* mutations result in altered petal size and shape

In order to separate the effects of *seu* and *lug* on petal shape from those on petal identity, we examined petal development in *seu ag* and *ag lug* double mutants. *AG* activity is not required for normal petal development (Bowman et al. 1989) and thus in the *ag* mutant background we can examine *seu* and *lug* petal shape defects without the confounding effects of organ identity transformations. The *ag* mutant makes petals in both whorls 2 and 3 (Bowman et al. 1989). We compared mature whorls 2 and 3 organs from the *seu-1 ag-1* and *ag-1 lug-1* mutant plants to those from the *ag-1* single mutant. The *ag-1* petals are wild type with respect to their size and shape (Fig. 1a, f) and epidermal cell surface morphology (Fig. 3a, d). Based on epidermal cell morphology three zones have been described along the apical–basal axis: the apical-located blade; the basally located claw; and a transition zone located between the blade and the claw (Fig. 1a) (Bowman 1994). The width of the *ag* petals averaged 1.2 mm (s.d.=0.2; $N=89$) at the widest extent of the petal blade. The *seu ag* (Fig. 1g) and *ag lug* (Fig. 1h) double mutants also form petals in both whorls 2 and 3 (Franks et al. 2002; Liu and Meyerowitz 1995). However, *ag lug* and *seu ag* petals are slightly, but significantly narrower than the *ag* petals [1.0 mm (s.d.=0.1; $N=41$) and 1.1 mm (s.d.=0.13; $N=25$), respectively], (Fig. 2). Thus *SEU* and *LUG* play a role in control of petal shape that is mediated by an *AG*-independent mechanism.

To further examine the *AG*-independent role of *SEU* and *LUG* in petal development, we analyzed the floral organs formed in whorls 2 and 3 of the *seu ag lug* triple mutant. These organs could be divided into two basic types based on overall morphology: narrow blade-shaped organs (Fig. 1b) and filamentous organs (Fig. 1c, d). Scanning electron microscopic analysis of epidermal cells indicates that both organ types display epidermal cells characteristic of petals (Figs. 1e, 3). Furthermore, cell types characteristic of other floral organs were not observed in these organs (data not shown). Thus we refer to these organs as blade-shaped petals and filamentous petals. The average width of the blade-shaped petals of the *seu ag lug* triple was 0.48 mm (s.d.=0.14; $N=44$), 40% the width of the *ag* petals (Fig. 2). The filamentous *seu ag lug* petals were very narrow averaging 0.12 mm (s.d.=0.05; $N=23$). This is about the width of the claw of the petal and thus represents almost no laminar expansion of the blade. Twenty-four percent of the whorls 2

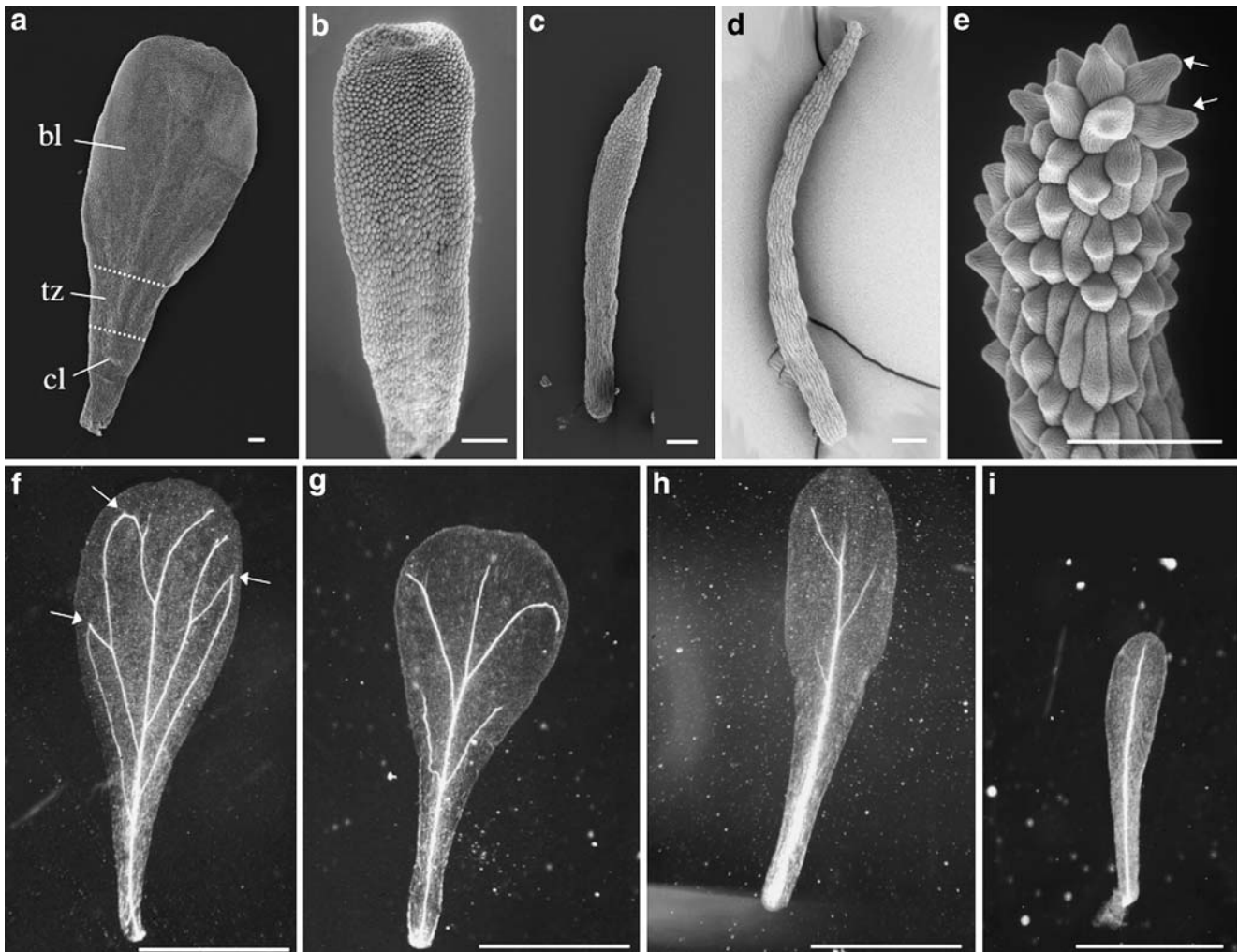


Fig. 1 Phenotypes of mature petals from *ag*, *seu ag*, *ag lug*, and *seu ag lug* mutants. **a–e** SEM micrographs. **f–i** Dark field images of optically cleared petals. **a** *ag-1* mutant petal with wild type size and shape. **b** Narrow blade-shaped petal from *seu ag lug* triple mutant. **c**, **d** Filamentous petals from *seu ag lug* triple mutant. **e** Tip of filamentous petal from *seu ag lug* triple mutant shows epidermal cell surface

morphology characteristic of petals (arrows). **f** *ag-1* petal with five secondary veins branching off of the primary vein. Three completed vascular loops are indicated (arrows). **g** *seu-1 ag-1* petal, **h** *ag-1 lug-1* petal. **i** *seu-1 ag-1 lug-1* blade-shaped petal. No secondary veins or vascular loops are present. Scale bars are 100 μ m in **a–d**; 50 μ m in **e**; and 1 mm in **f–i** (*bl* petal blade, *tz* transition zone, *cl* petal claw)

and 3 organs examined (35 of 144) from *seu ag lug* triple mutant flowers were filamentous. A smaller percentage of filamentous organs (2 of 27) was observed in the *seu ag* double mutants. Filamentous petals were not observed in the *ag-1* mutants (0 of 89), nor in the *lug-1 ag-1* double mutants (0 of 41). Thus, *seu* and *lug* mutants display a synergistic effect on petal shape as evidenced by increased prevalence of filamentous petals and enhanced narrowing of blade-shaped petals in the *seu ag lug* flowers. The effect of *SEU* and *LUG* on the shape of lateral organs was not restricted to petals, as narrowing of leaves and sepals is also observed (data not shown).

We also examined petal length in these genotypes. The length of *ag* petals, measured from the base of the claw to the tip of the blade, averaged 3.4 mm (s.d.=0.25; $N=89$) (Fig. 2). The *seu ag* petals were slightly shorter than the *ag* petals averaging 3.1 mm (s.d.=0.21; $N=24$), while the *ag lug* petals were slightly longer than the *ag*

petals averaging 3.7 mm (s.d.=0.16; $N=41$). Petal length in the *seu ag lug* blade-shaped petals was significantly reduced relative to *ag* petals, to 2.5 mm (s.d.=0.44; $N=44$). Filamentous petals from the *seu-1 ag-1 lug-1* triple mutants displayed the greatest reduction in length averaging 1.6 mm (s.d.=0.23; $N=23$).

seu and *lug* mutants display a reduction of petal vascular elements

We analyzed the petal vascular elements in mature cleared petals under dark field illumination (Fig. 1f–i). Visual inspection of *seu ag*, *ag lug* and *seu ag lug* petals suggested that the extent of vascularization in these mutants was reduced when compared to that of *ag* mutants. To quantify petal vascularization we counted the number of secondary veins that branched off the primary central vein. We also counted intact vascular loops

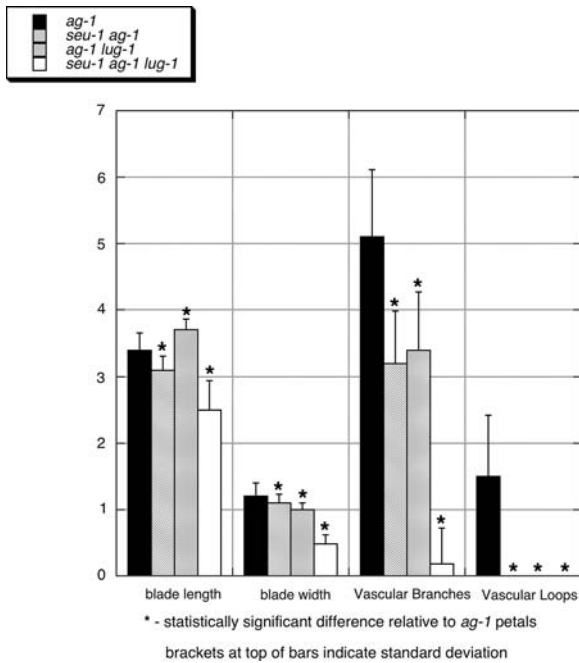


Fig. 2 Average petal length, width and vasculature measurements for blade-shaped petals. *Brackets* at the top of each bar indicates the standard deviation

(Fig. 1f, arrows). The *ag-1* petals displayed on average 5.1 (s.d.=1.0; $N=89$) secondary veins per petal (Fig. 2). The *seu-1 ag-1* and *ag-1 lug-1* double mutants displayed significantly fewer secondary veins, 3.2 (s.d.=0.78; $N=25$) and 3.4 (s.d.=0.87; $N=41$) per petal, respectively. The *seu ag lug* triple mutants displayed a more severe petal vascular defect showing on average 0.18 (s.d.=0.5; $N=44$) secondary veins per petal. The absence of *SEU* and *LUG* activities also led to a significant reduction in the number of vascular loops. The *ag-1* petals displayed 1.5 (s.d.=0.9; $N=89$) loops per petal, while no intact vascular loops were observed in the *seu ag* ($N=25$), *ag lug* ($N=41$) or *seu ag lug* ($N=44$) mutant petals. These analyses included the blade-shaped *seu ag lug* triple mutant petals but excluded the filamentous *seu ag lug* petals. The primary central vein is most often absent in the *seu ag lug* filamentous petals and we never observed secondary veins, nor loops in these filamentous petals ($N=23$).

Reduced petal blade size results from a reduction of cell number

We compared the area of the petal blade between *ag* mutant petals and the blade-shaped *seu ag lug* petals. The filamentous petals were excluded from this analysis as they contain almost no blade. The petal blade area averaged $0.96 \pm 0.14 \text{ mm}^2$ in the *ag* mutant petals as compared to $0.19 \pm 0.11 \text{ mm}^2$ in the *seu ag lug* blade-shaped petals (Table 1). The fivefold reduction in blade area observed in the *seu-1 ag-1 lug-1* triple mutants could be caused by a reduction in cell number, cell size or both. To

distinguish between these possibilities, we measured the cross-sectional area of epidermal cells within the petal blade. The average cross-sectional area of adaxial petal epidermal cells was $2.3 \times 10^{-4} \text{ mm}^2$ in the *ag* mutant and was $2.1 \times 10^{-4} \text{ mm}^2$ in the *seu ag lug* triple mutant (Table 1). Thus, the cells of the *seu ag lug* triple mutant display only a slight reduction in size relative to the *ag* mutant cells. Using the observed average cell sizes we calculated the total number of petal blade epidermal cells for the adaxial surface. The *seu-1 ag-1 lug-1* petal blades showed over fourfold fewer epidermal cells (910) than the *ag-1* mutants (4,170) (Table 1). Similar results were obtained for the abaxial surface (data not shown). The data indicate that the reduction in petal blade area in the *seu-1 ag-1 lug-1* mutant is largely the result of reduced cell number.

Epidermal cell morphology and ectopic lamina indicate loss of polarity in *seu ag lug* petals

We reasoned that the reduction in laminar growth displayed in the *seu ag lug* petals could be caused by a failure to properly specify or maintain adaxial/abaxial polarity. To test this hypothesis we examined the surface morphology of petal epidermal cells. In wild type petals, as well as the *ag* mutant petals, the adaxial epidermal cells are cone-shaped and have many epicuticular ridges running from the base to the apex of the cone (Bowman 1994) (Fig. 3d). These ridges are mostly straight. In contrast, the abaxial epidermal cells are more rounded and cobblestone-shaped with wavy epicuticular ridges (Fig. 3a). We examined the epidermal cell morphology of *seu-1 ag-1 lug-1* triple mutant petals. Results from the blade-shaped petals and the filamentous petals were somewhat different. Blade-shaped petals displayed relatively normal epidermal cell morphology on their abaxial surfaces (Fig. 3b). However, the adaxial surfaces of the blade-shaped petals were variably affected. In about half of the cases, the typical adaxial cell surface morphology was observed (data not shown) while in the other cases cells of a more intermediate form, with an enhanced waviness of the epidermal cuticle ridges, was observed (Fig. 3e). In these cases, the adaxial epidermal cells appeared to be partially abaxialized. The most severe alterations of cell morphology were seen in the filamentous petals (Fig. 3c, f). These petals often displayed a

Table 1 Average cell size and average number of cells per petal blade for *ag* single and *seu ag lug* blade-shaped petals

	Adaxial petal blade area (mm^2)	Cell size ($\times 10^{-4} \text{ mm}^2$)	Adaxial epidermal cells per blade
<i>ag-1</i>	0.96 ± 0.14 $N=15$	2.3 ± 0.5 $N=165$	4,170
<i>ag-1/seu-1/lug-1</i>	0.19 ± 0.11 $N=15$	2.1 ± 0.7 $N=135$	910

Values are average \pm s.d.

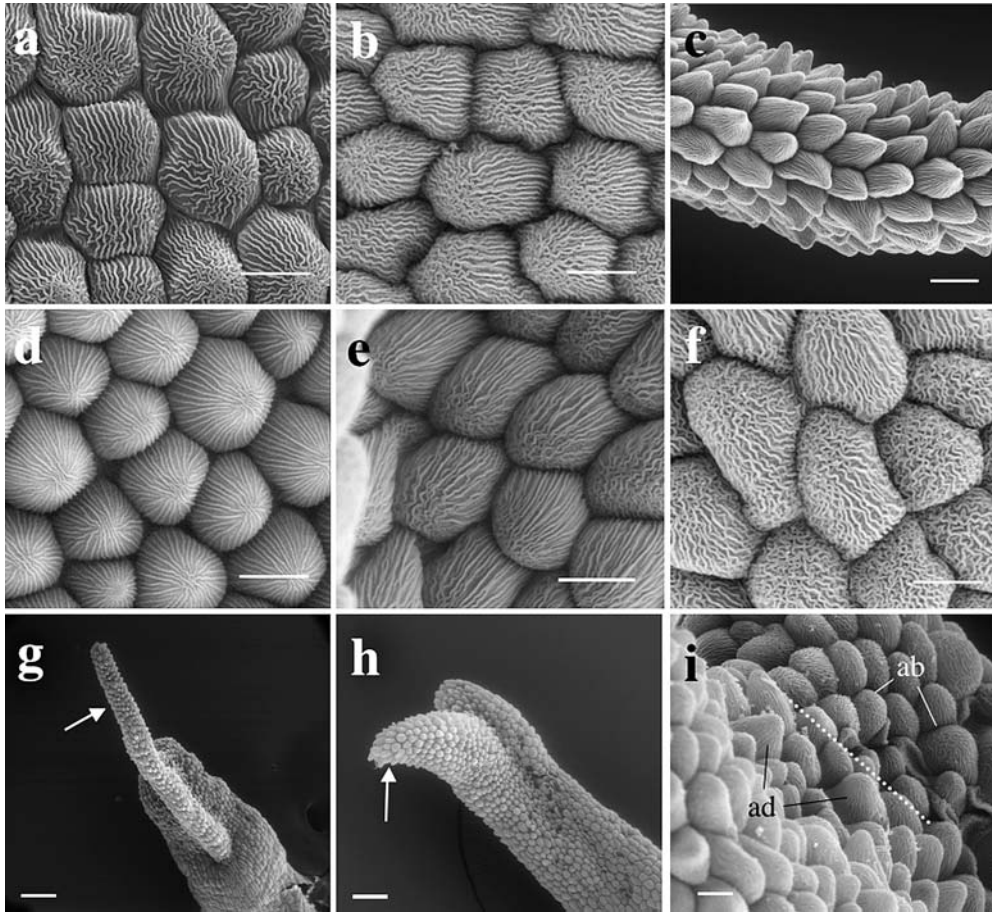


Fig. 3 SEM micrographs of petal epidermal cell surface morphology and ectopic lamina/filament. **a** *ag-1* abaxial petal surface. Cells have cobblestone shape and wavy epicuticular ridges that are characteristic of abaxial petal cells. **b** *seu-1 ag-1 lug-1* abaxial petal surface (blade-shaped petal). Cells exhibit normal abaxial features. **c** *seu-1 ag-1 lug-1* filamentous petal. Cells exhibit mainly adaxial cell morphology (i.e. cone shape and straight epicuticular ridges). This filament appears adaxialized around its entire surface. **d** *ag-1* adaxial petal surface. Cells are cone-shaped and exhibit straight epicuticular ridges along conical axis of the cells. **e** *seu-1 ag-1 lug-1* adaxial petal surface (blade-shaped petal). Cells exhibit partially rounded shape

and some waviness of ridges. Cells appear partially abaxialized. **f** *seu-1 ag-1 lug-1* adaxial surface (filamentous petal). Cells exhibit abaxial characteristics indicating abaxialization of this petal. **g** *seu-1 ag-1 lug-1* petal with ectopic filament (arrow) arising from the abaxial surface of the primary petal. **h** *seu-1 ag-1 lug-1* petal. Tip of petal exhibits an ectopic blade lamina (arrow) that is forming, thus defining a new axis of growth. **i** Close-up view of the base of the ectopic lamina shown in (**h**). A juxtaposition of adaxial (*ad*) and abaxial (*ab*) cell types is observed. Scale bars 10 μ m in (**a**), (**b**), (**d-f**), and (**i**); 20 μ m in (**c**); 100 μ m in (**g**); and 50 μ m in (**h**)

predominance of one cell type around the entire circumference of the filamentous petal. Examples of entirely adaxialized petals (Fig. 3c), as well as entirely abaxialized (Fig. 3f) petals were found.

Occasionally, the small blade lamina that had developed at the apex of the filamentous petal was mis-orientated relative to the plane of the petal claw (data not shown). We also observed instances where a secondary ectopic filament (Fig. 3g) or blade (Fig. 3h) had formed at an offset angle relative to the plane of the primary blade. Cells at the base of these ectopic lamina were a mixture of adaxial and abaxial cell types (Fig. 3i), suggesting that these lamina may arise from a zone of juxtaposition between adaxial and abaxial cell types. Thus, epidermal cell morphology and the formation of ectopic lamina suggest a mis-specification of adaxial and abaxial fates in the *seu ag lug* mutant petals.

The *seu ag lug* triple mutant petals display reduced levels of the adaxial and abaxial identity regulators, *PHB* and *FIL/YABI*

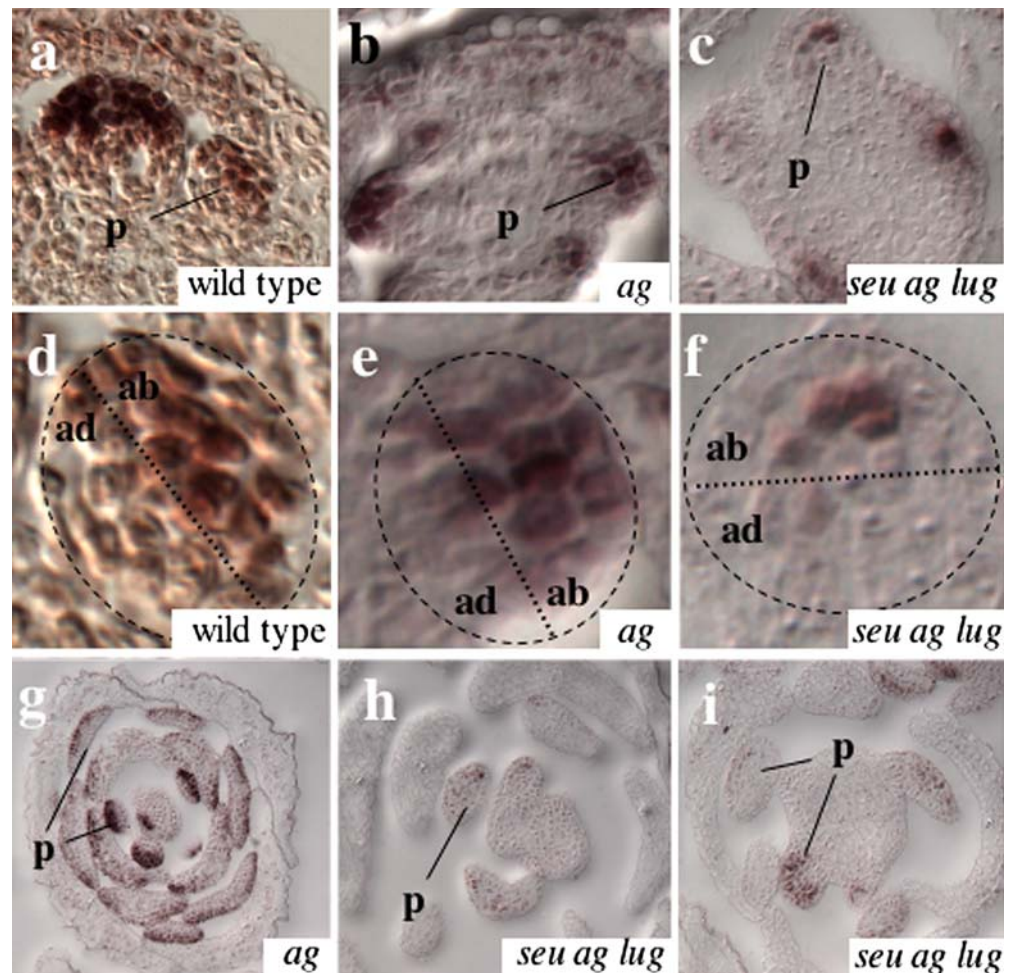
To identify the underlying mechanisms of the polarity defects observed in the *seu ag lug* mutant petals, we examined the expression of two key regulators of adaxial/abaxial polarity in the lateral organs, *PHB* and *FIL/YABI* (McConnell et al. 2001; Sawa et al. 1999; Siegfried et al. 1999). *PHB* and *FIL/YABI* specify adaxial and abaxial polarity in the lateral organs, respectively. Additionally, their mRNAs accumulate specifically in the adaxial and abaxial petal domains and thus they serve as useful markers of these spatial domains (Prigge et al. 2005; Sawa et al. 1999; Siegfried et al. 1999); (Figs. 4, 5). If *LUG* and *SEU* act upstream of *PHB* or *FIL/YABI* accumulation, we expect to observe a

reduction or mis-expression of *PHB* or *FIL/YABI* mRNAs within the *seu* or *lug* mutant petal. However, if *LUG* and *SEU* act downstream of *PHB* or *FIL/YABI*, the expression of the latter might not be affected. As the expression of these markers had not been fully characterized throughout wild-type petal development, we used in situ hybridization to determine the spatial localization of the *FIL/YABI* and *PHB* mRNAs in wild type, *ag* and *seu ag lug* petal primordia.

In wild type stage 5 flowers (floral stages according to Smyth et al. 1990), *FIL/YABI* expression was detected throughout the petal primordia just after they arise (data not shown). During stage 6, *FIL/YABI* is detected in a more polarized pattern with highest expression in the abaxial-most three petal cell layers (Fig. 4 a, d). Lower levels of expression are detectable in the adaxial half of the primordia. Expression in adaxial portions of the petal was not detected above background in our experiments after floral stage 6. Expression in petals continues to be detected in the abaxial-most two or three cell layers from stage 6 until at least floral stage 10 (data not shown). In *ag-1* petals from stage 6 flowers, *FIL/YABI* expression is detected most strongly in the abaxial-most three cell layers in a pattern similar to that seen in wild

type petals (Fig. 4 b, e). In contrast, analysis of the *seu-1 ag-1 lug-1* triple mutant petals revealed that the expression of *FIL/YABI* was greatly reduced or absent in developing petal primordia. In *seu ag lug* stage 6 flowers, expression is detected at low levels or detected in only a few cells within the abaxial portion of the petal (Fig. 4 c, f). This is in contrast to the strong expression throughout the entire abaxial portion of wild type and *ag* mutant petals at this stage. Because *ag* mutants fail to generate stamens and carpels, it is impossible to determine the exact stage of each petal primordia in the *ag* and *seu ag lug* mutant flowers. However, it was possible for us to estimate the developmental stage of petals in these genotypes based on the number of organ whorls that had initiated and the distance of the organ from the floral meristem axis. In older *seu ag lug* triple mutant petals, expression of *FIL/YABI* was either not detected or was observed to be more patchy and diffuse than that seen in wild type or *ag* mutant petals of a similar developmental stage (compare Fig. 4g to Fig. 4h, i). In summary our data indicate that the *seu ag lug* triple mutant petals displayed reduced levels of *FIL/YABI* expression throughout their development relative to the wild type and *ag* mutant petals.

Fig. 4 Expression of abaxial fate determinant *FIL/YABI* is reduced in the *seu ag lug* triple mutant petals. Floral cross sections hybridized with *FIL/YABI* antisense in situ probe. **a, d** Expression is detected most strongly in abaxial portions of the wild type (Columbia) stage 6 petal primordium. **b, e** Expression in *ag* mutant petal (equivalent to stage 6) is detected in abaxial portion of primordium. **c, f** Expression in the abaxial portions of *seu ag lug* petal primordium is detected in fewer cells than in *ag* mutant. Ovals in (**d-f**) indicate approximate extent of petal primordium. **g** Older petals from *ag* mutant flowers continue to express *FIL/YABI* within the two or three abaxial-most cell layers. **h, i** Expression of *FIL/YABI* in *seu ag lug* triple mutant petals is very reduced in older petals. *ab* abaxial, *ad* adaxial, *p* petal



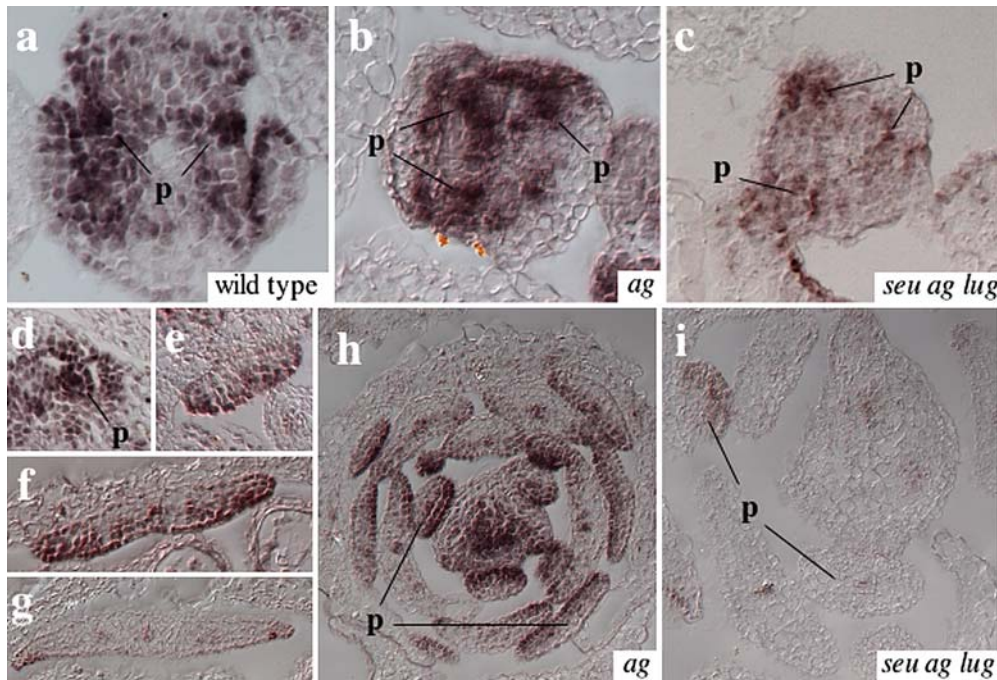


Fig. 5 Expression of adaxial fate determinant *PHB* is reduced in the *seu ag lug* triple mutant petals. Floral cross sections hybridized with *PHB* antisense in situ probes. **a** Expression is detected throughout petal primordia in wild type (Ler) stage 5 flower. **b** Expression is detected throughout petal primordia in *ag* mutant flower (equivalent to stage 5). **c** Expression in *seu ag lug* mutant petal primordia appears diffuse and reduced in intensity relative to the *ag* mutant. **d** Expression is detected throughout wild type stage 6 petal primor-

dium. **e** In a late stage 8 wild type petal, expression is detected in gradient throughout petal with highest expression in the adaxial-most three cell layers. **f** In a stage 10 wild type petal, strong expression in adaxial most three cell layers is evident. **g** In a stage 11 wild type petal, expression is largely confined to adaxial margins and vasculature. **h** Expression in late stage *ag* mutant petals is maintained in pattern similar to that seen in wild type. **i** Expression in late stage *seu ag lug* triple mutant petals is very reduced relative to *ag* mutant petals

Using an antisense *PHB* in situ probe we detected expression throughout stage 5 wild type petal primordia (Fig. 5a). In petals from stage 5 *ag-1* flowers, *PHB* expression is also detected throughout the extent of the primordia (Fig. 5b). However, analysis of petals from similarly staged *seu-1 ag-1 lug-1* triple mutant flowers revealed that *PHB* expression was patchy and diffuse and the expression level appeared reduced relative to wild type or *ag* mutants (Fig. 5c). In wild type petal primordia, *PHB* expression continues to be expressed through out the entire petal primordia during floral stage 6 (Fig. 5d). This expression pattern is later refined and expression is detected most strongly within the adaxial-most three cell layers during floral stages 8–10 (Fig. 5f, g). *PHB* expression is still detected in petals from stage 11 flowers, but is limited to the adaxial margins of the petal (Fig. 5g). *PHB* expression is detected most strongly in the adaxial-most three cell layers in late stage petals from *ag* mutant flowers (Fig. 5h) in a pattern similar to that seen in wild type flowers. In contrast, expression of *PHB* in late stage *seu ag lug* triple mutant petals was often not detected or was detected at very low levels (Fig. 5i). Taken together these results suggest that the molecular specification of adaxial/abaxial polarity in the *seu ag lug* triple mutant petal is compromised.

Discussion

SEU and *LUG* are required for complete specification of adaxial and abaxial petal identities

Several observations suggest that *SEU* and *LUG* are required for the proper and complete specification of polarity along the adaxial/abaxial axis of the developing petal. (1) The analysis of epidermal morphology of petal cells revealed cases of altered fate specification. Filamentous petals were often observed to contain just one surface cell type within the extremely reduced blade. Examples of either complete adaxialization (Fig. 3c) or complete abaxialization (Fig. 3f) were detected. Alterations in the surface-specific pattern of epidermal cell morphology were observed in the blade-shaped *seu ag lug* petals, but were generally less severe than those observed in the filamentous petal. (2) Expression of *PHB* and *FIL/YABI* was found to be reduced in the developing *seu ag lug* petal primordia, suggesting a reduction of (although not a complete loss of) the specification of both adaxial and abaxial fates in this triple mutant. (3) Ectopic laminae were observed on the surface of the *seu ag lug* mutant petals. The formation of ectopic laminae has been reported in maize (Timmermans et al. 1998)

and *Arabidopsis* leaves (Eshed et al. 2004; Prigge et al. 2005) as a result of compromised polarity specification along the abaxial/adaxial axis.

Mechanisms of petal shape regulation

Our analysis indicates that the petals of the *seu ag lug* triple mutants contain one-fourth to one-fifth of the wild type number of petal blade cells. As the continued expression of YABBY family members has been shown to be important for the continued laminar expansion of the leaf blade (Eshed et al. 2004), *SEU* and *LUG* may function through *PHB* or *FIL/YABI* to stimulate cellular division during lamina outgrowth. Our data indicate that reductions in *PHB* and *FIL/YABI* expression are evident early during petal development (from floral stage 5 and 6, respectively), before the petal primordia show obvious morphological alterations. This data argues against a model in which *SEU* and *LUG* control the initial number of primordia founder cells and supports a model in which *SEU* and *LUG* are required for the division of cells within the primordium after their initial recruitment.

In *seu ag lug* stage 6 flowers, we observed expression of *FIL/YABI* in fewer cells within the petal primordia relative to wild type, but this expression was properly localized to the abaxial portion of the primordia. Similarly, *PHB* expression when observed in the *seu ag lug* triple mutant petals was weaker and more diffuse relative to that seen in wild type petals, but was typically detected within the adaxial portion of the primordium. These results indicate that patterning of the adaxial and abaxial domains is not completely disrupted in the *seu ag lug* triple mutant. At least two models are consistent with the data. One proposes that *SEU* and *LUG* are required for the generation or reception of a polarizing signal required for petal adaxial/abaxial polarity specification. In this model the lower level of polarizing activity in the *seu ag lug* mutant results in fewer cells of the primordia being properly specified with respect to adaxial/abaxial identity. Alternatively, *SEU* and *LUG* may not affect the level of the polarizing signal directly, but rather may be more indirectly required for cellular responses within the primordia that are necessary to refine and maintain the initial response to the polarizing signal. The low levels of expression of *PHB* and *FIL/YABI* within late stage *seu ag lug* mutant petals suggest that *SEU* and *LUG* are required throughout petal development to reinforce and maintain the expression of *PHB* and *FIL/YABI*. Thus *SEU* and *LUG* may not establish abaxial and adaxial domains specifically, but may potentiate the action of *PHB* and *FIL/YABI* during the maintenance of the polarity states.

As transcriptional co-regulators, *SEU* and *LUG* are thought to require interaction with a DNA-binding protein to mediate transcriptional regulation. One candidate for this DNA binding activity is *FIL/YABI* which has been shown to bind DNA in vitro in a non-sequence specific manner (Kanaya et al. 2002). Work in *Antirrhinum*

indicates the existence of orthologues of *SEU* and *LUG* whose molecular action during *Antirrhinum* floral development appears to be quite similar (Navarro et al. 2004). *STYLOSA* (*STY*), the *Antirrhinum* orthologue of *LUG*, has been shown to physically interact with *Antirrhinum* SEUSS-LIKE proteins (*AmSEU1*, *AmSEU2*, *AmSEU3*) as well as the *Antirrhinum* *FIL* orthologue, *GRAMINIFOLIA* (*GRAM*) suggesting that *LUG* and *SEU* may bind directly to *FIL/YABI* in *Arabidopsis* (Navarro et al. 2004). Furthermore, the *Antirrhinum sty gram* double mutants display enhanced loss of leaf and petal polarity suggesting that this protein complex regulates polarity specification and laminar outgrowth in *Antirrhinum*. In this scenario, *SEU*, *LUG* and *FIL/YABI* would be required in the petal for the maintenance of *FIL/YABI* expression that has been shown to be important for laminar outgrowth in leaf (Eshed et al. 2004). Although evidence for autoregulation of *FIL/YABI* has not yet been reported, *INNER NO OUTER* (*INO*), a member of the YABBY gene family is known to positively autoregulate its own expression during the development of the outer integument (Meister et al. 2002, 2005).

A second candidate DNA binding activity to tether the *SEU/LUG* complex is the *AINTEGUMENTA* (*ANT*) protein. *ANT* encodes a DNA-binding transcriptional regulator of the *AP2* family of proteins (Elliott et al. 1996; Klucher et al. 1996). *ANT* mRNA is expressed within the developing petal blade lamina (Elliott et al. 1996) and known to be required for laminar growth (Elliott et al. 1996; Klucher et al. 1996; Krizek 1999; Mizukami and Fischer 2000). The *seu*, *lug* and *ant* mutants display a variety of genetic interactions supporting a close functional association between these three genes (Krizek et al. 2000; Liu et al. 2000). In addition, expression of *PHB* is reduced in *fil ant* double mutant flowers (Nole-Wilson and Krizek, personal communication). Nole-Wilson and Krizek have also demonstrated that *ANT* binds to a conserved DNA element in the *YABI* and *YAB3* promoters, although a change in the levels of *YABI* or *YAB3* RNA was not detected in *ant* mutant flowers.

SEU and *LUG* regulate vascular development

The *seu ag lug* triple mutants display a dramatic reduction in petal vascularization. One model suggests that the loss of *PHB* or *FIL/YABI* expression observed in the *seu ag lug* mutant petals may be responsible for the vascular patterning defects. A reduction in the vascular branching was observed in *yab1 yab3* double mutant cotyledons (Siegfried et al. 1999). An alternative but not mutually exclusive model proposes that an altered sensitivity to the plant hormone auxin in the *seu ag lug* mutants is responsible for the reduction in petal vascular elements. Vascular elements are thought to be patterned by and to require the directional flow of auxin in sites of future vascular development (Nelson and Dengler 1997). Previously *seu* mutants were reported to have reduced vasculature in cotyledons and to display a reduced sensitivity

to exogenous auxin (Pfluger and Zambryski 2004). In *Antirrhinum*, a loss-of-function mutant in the putative *LUG* orthologue, *STYLOSA*, shows altered responses to exogenous auxin and to the auxin transport inhibitor NPA and defects in vein patterning (Navarro et al. 2004). Thus *SEU* and *LUG* may both be required for responses to auxin during petal vascular development.

Acknowledgments We thank John Bowman for providing *PHB* and *YAB1/FIL* in situ probes; Yuki Mizukami for suggestions regarding petal cell size analysis; Sridevi Ashakanandam for technical assistance; undergraduate researchers Sarah Stefanos and Bill Tran; and the UC Berkeley and NCSU Centers for Electron Microscopy. We thank Jose Alonso, Anna Stepanova, Beth Krizek, Tzung-Fu Hsieh, and anonymous reviewers for commenting on the manuscript. This work was funded by the following grants: NSF IOB-0416759, USDA ARS NC06759 and NIH NRSA Postdoctoral Fellowship GM20426-02 to R.G.F.

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