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The role of *SEUSS* in auxin response and floral organ patterning

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Summary

Genetic and physiological analyses implicate auxin flux in patterning, initiation and growth of floral organs. Within the *Arabidopsis* flower, the *ETTIN/ARF3* transcription factor responds to auxin to effect perianth organ number and reproductive organ differentiation. This work describes a modifier of *ettin* that causes filamentous, mispositioned outer whorl organs and reduced numbers of malformed stamens in the double mutant. The modifier was discovered to be a new allele of the *seuss* (*seu*) mutant. *SEU* encodes a novel protein that is predicted to transcriptionally co-repress the *AGAMOUS* floral organ identity gene. The effects of *seu* on *ett* are shown to be independent of the *SEU-AG* pathway. Furthermore, morphological, physiological and genetic evidence implicate *SEU* in auxin-regulated growth and development. *seu* has a pleiotropic phenotype that includes reductions in several classic auxin responses such as apical dominance, lateral root initiation, sensitivity to exogenous auxin and activation of the DR5 auxin response reporter. *seu* displays a synergistic interaction with the auxin response mutant *pinoid*, producing flowers with few outer whorl organs. Collectively, these data suggest that *SEU* is a novel factor affecting auxin response. A model is proposed in which *SEU* functions jointly with *ETT* in auxin response to promote floral organ patterning and growth.

Key words: Seuss, Auxin, Ettin, Flower, Pinoid

Introduction

Among plant hormones, auxins are well known for their widespread role in growth and development. The most biologically active of these simple molecules is indole-acetic acid (IAA), a significant proportion of which is synthesized in young actively dividing cells in the aerial part of the plant. IAA is transported in a polar fashion throughout the plant to affect a variety of morphological processes (Davies, 1995). At the cellular level, auxin regulates cell division and elongation, which explains its essential role in developing tissues (Sachs, 1991). Auxin contributes to such fundamental processes as embryonic axis establishment, organization of the root meristem and subsequent root elongation, and lateral organ outgrowth in both the root and the shoot (Friml et al., 2003; Jiang and Feldman, 2002; Casimiro et al., 2003; Reinhardt et al., 2000). Auxin continues to influence development throughout the life of the plant. During flowering, it promotes organ outgrowth and development of the gynoecium, the female reproductive organ of angiosperms (Vernoux et al., 2000; Nemhauser, 2000; Okada et al., 1991; Bennett et al., 1995).

The *Arabidopsis* flower is particularly well suited to study the effects of auxin on plant development because the flower arises in a very stereotypical pattern, resulting in four types of organs that are morphologically distinguishable from one another. Each organ type develops in a concentric whorl: four evenly spaced sepals envelop and protect the developing internal organs; four petals arise just inside the sepal margins; six stamens are patterned as two lateral and two pairs of medial organs; and two fused carpels collectively comprise the central gynoecium. The conceptual framework for flower development has been the elegantly simple ABC model, in which three classes of genes are expressed in concentric overlapping domains that combinatorially specify organ identity in each of the four floral whorls (Bowman et al., 1991). Although identification of genes involved in meristem function and organ identity has increased exponentially (Carles and Fletcher, 2003; Franks and Liu, 2001), relatively little is known about how organs are patterned both within and between whorls. Transcription factors such as SUPERMAN and CUP-SHAPED COTYLEDON2 function in whorl and/or organ partitioning (Aida et al., 1997; Sakai et al., 1995), while UNUSUAL FLORAL ORGANS and PERIANTHIA affect initiation and spacing of floral organs (Levin and Meyerowitz, 1995; Running and Meyerowitz, 1996). Yet how floral organs are initiated in such specific positions and in such a reproducible pattern is still not well understood.

The *ettin* (*ett*) mutant has pleiotropic effects on flower development, including increases in perianth organ number and aberrations in regional differentiation of reproductive organs. In *ett*, development of the gynoecium has been the best characterized. The gynoecium in the *Brassicaceae*, including *Arabidopsis*, develops from two congenitally fused carpels that arise from the center of the floral meristem. The two outer ovary walls, or valves, are separated from each other by a longitudinal furrow, or replum. In *ett*, the organization of apical style and stigma, central ovary and basal internode (termed stipe), is disrupted. Strong alleles of *ett* are characterized by a shift in boundaries between these tissues, including a reduction in valve length, an increase in basal stipe, and an overproliferation of apical stigma and style (Sessions and Zambryski, 1995). There is also a concomitant eversion or

abaxialization of internal tissues, the severity of which increases towards the gynoecium apex. The reduction in the proportion of ovary in *ett* is reminiscent of the phenotypes of the auxin regulatory mutants *pinoid* (*pid*) and *monopteros* (*mp*), and therefore similarly may be due to a disruption in auxin signaling (Bennett et al., 1995; Przemeck et al., 1996). In fact, transient application of naphthylphthalamic acid (NPA), a polar auxin transport inhibitor, to the inflorescence apex of wild-type plants results in a reduction of ovary relative to apical style and basal stipe, effectively phenocopying *ett* and weak alleles of *pid*. Because NPA presumably causes auxin to pool at its sites of synthesis instead of being transported in a normal polar fashion, it has been hypothesized that ETT responds to a gradient of auxin to establish ovary boundaries within the gynoecium (Nemhauser et al., 2000).

ETT is a member of the auxin response factor (ARF) family of transcription factors (Sessions et al., 1997). This family is central to auxin response, as almost immediately after auxin entry into a cell, ARFs activate transcription of early auxin response genes (Ulmasov et al., 1999a). The ARF family is difficult to study genetically, owing to a high level of sequence homology and probable functional redundancy. Only three out of 22 ARFs from *Arabidopsis* have demonstrated loss-of-function phenotypes (Guilfoyle and Hagen, 2001). ETT is unique among ARFs because it lacks two C-terminal domains responsible for heterodimerization with Aux/IAAs, a family of transcriptional repressor proteins. Instead, ETT has a unique C-terminal half, containing a region rich in serine residues. Thus, the prevailing model for auxin action – in which Aux/IAA repressors are rapidly targeted for degradation when auxin is present, liberating ARFs to activate transcription of target genes – cannot hold for ETT. Yet ETT is clearly involved in auxin response in the gynoecium, as NPA treatment of wild type gynoecia phenocopies *ett*. In addition, auxin may have an as yet undescribed role in outer whorl development, because the auxin response mutant *ett*, the auxin signaling mutant *pid*, and the auxin transport mutant *pin-formed1* (*pin1*) all alter floral organ numbers (Bennett et al., 1995; Sessions, 1997).

To understand more about how ETT responds to auxin to influence initiation and patterning of floral organs, modifiers of the *ett* phenotype were identified. One such modifier was found to carry a mutation in the *SEUSS* (*SEU*) gene, which encodes a putative transcriptional co-regulator of the floral homeotic gene *AGAMOUS* (*AG*) (Franks et al., 2002). We describe the effects of *seu* on *ett* and extend genetic and morphological studies using the novel *seu-3* allele. We demonstrate that *seu* confers auxin resistance to the root and disrupts flower development in combination with the auxin response mutants *ett* and *pid*. We propose a model in which SEU acts in concert with ETT to promote floral organ development by transcriptionally regulating auxin-responsive genes.

Materials and methods

Genetic stocks

Seeds of *seu-1/+ ag-1/+* are courtesy of Bob Franks. The following seeds have been deposited at the ABRC: *seu-3* in Col-0; *seu-3* in Ler-0; *ett-7* in Col-0; *ett-11* in Ler-0. *seu-3* was originally identified by Allen Sessions and Judy Roe as *s27* from an EMS-mutagenized population of Col seeds. *seu-4* is a T-DNA insertion line from the Salk Institute Genomic Analysis Laboratory; the ABRC stock number is SALK_069303. *seu-3* CAPS genotype markers can be generated by PCR with forward primer GAAAATGTTCCGCCTTCGAT (+40 bp past the start of translation in *SEU*) and reverse primer GAATTTGCTGCGGTTCCAACT (beginning at *SEU* +619). *Bsl*I or *Pfl*MI cleaves wild-type DNA to yield 235 and 345 bp fragments, whereas in *seu-3* the uncleaved product is 580 bp. Alternatively, *Bsr*I can be used to generate wild-type fragments of 241 and 339 bp. The *seu-4* allele can be identified with forward primer TGGACCGCTTGCTGCAACT (235 bp inside the T-DNA left border of pROK2, the vector used to generate the SALK lines) and reverse primer CCGGCATTTGAAGAAATTGT (at *SEU* +742), which generate a 264 bp PCR product. T-DNA left border (110 bp) is missing in the *seu-4* allele, and there is 8 bp of homology at the insertion point between the left border and *SEU*.

Plant growth conditions

Plants grown on plates were in a growth chamber with 24 hours light at 22°C. Culture media consisted of half-strength MS salts, 1% sucrose and 0.8% bacto-agar at pH 5.8. For naphthaleneacetic acid (NAA) experiments, NAA was dissolved in 70% ethanol and added to the medium before autoclaving, or in 1 M NaOH and added after autoclaving. Plants grown in soil were sown on Metromix in 7.6 cm^2 pots and grown at a 1-4 per pot density under long-day greenhouse conditions.

Root assays

seu and wild-type seeds were grown on vertical plates. At 1-2 days after planting (dap), seedlings were examined for presence of an emerged radicle, and these seedlings were transplanted at 3 dap to treatment plates (100 mm^2). *seu* and wild-type seedlings were grown on the same treatment plate. For lateral root counts, roots were counted at 10 dap. For measurements of primary root length, digital photos were taken at 8 dap and roots measured in ImageJ (public domain software available from NIH at http://rsb.info.nih.gov/ij/). The segmented line tool was used to trace roots, and the measure function was used to determine root length. For root reorientation assays, plates were rotated 90° with respect to gravity and photos were taken 24 hours later. Root angles were calculated in Adobe Photoshop using the measure tool.

Histochemistry

β-Glucuronidase staining was performed using 2 mM X-gluc substrate from Rose Scientific, 2 mM potassium ferrocyanide, and 2 mM potassium ferricyanide, at room temperature from 3 hours to overnight (Weigel and Glazebrook, 2002).

Yeast two hybrid assays

Yeast two hybrid protocols are from Clontech. SEU prey is in pGAD424, ETT bait is in pBD, ARF1 bait is in pGBT9 and UFO bait is in pAS1. pBD and pGAD424 were used as empty vector controls. All assays were performed in yeast strain YD116.

Results

Identification of a strong *seuss* allele

The *seu-3* mutant characterized in this study was originally identified as *s27* in a screen for floral mutants (R. A. Sessions, PhD Thesis, University of California, Berkeley, 1996). Because of its synergistic double mutant phenotype with *ett* (see below), map-based cloning was begun. *s27* mapped close to GAPB on chromosome I, near the *SEU* gene (Franks et al., 2002). Based on the comparable gynoecium defects in both mutants, we tested allelism, and all descendants from the selfed progeny of a complementation cross had a *seu*-like phenotype. Thus, *s27* was renamed *seu-3*. The *SEU* gene in *seu-3* was sequenced and a C-to-T transition was found at amino acid 127

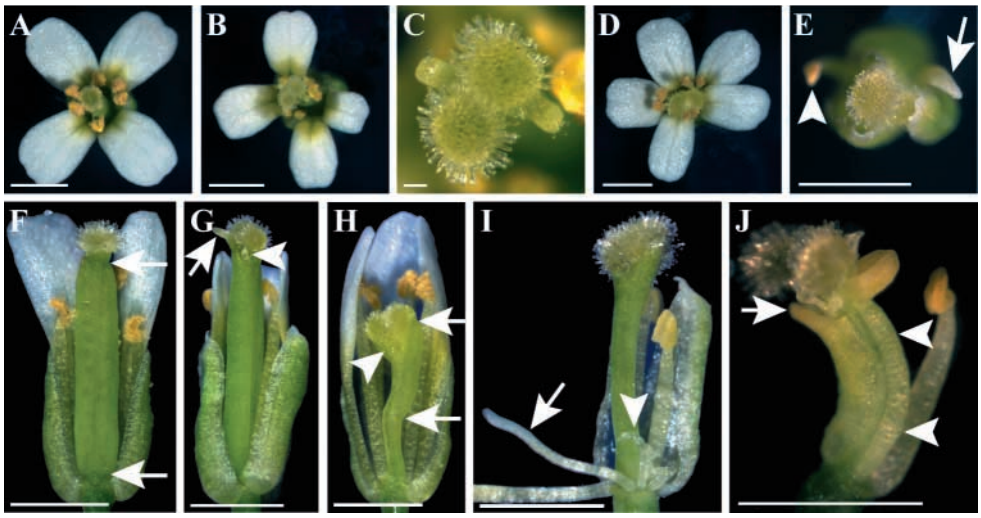

Fig. 1. *seu-3* modifies *ett-7* in all floral whorls. (A-E) Top-down views. (F-J) Side views. (A) Wild-type flower showing four evenly spaced petals, six stamens and the stigma at the top of the central gynoecium. (B) *seu-3* flower showing smaller, narrower petals, shorter stamens and a split gynoecium. (C) Close up of *seu-3* gynoecium in B showing two balls of stigmatic tissue and two splayed valve tips. (D) *ett-7* flower showing five petals, six stamens and a wide stigma resulting from overproliferation of apical tissues. (E) *ett-7 seu-3* flower showing one very narrow petal (arrow), one stamen (arrowhead) and a round stigma. (F) Wild-type flower showing two full ovaries, with arrows delimiting top and bottom of the right valve. (G) *seu-3* flower showing smaller petals and shorter pollenless stamens. Ovaries are full length, but the unfused distal gynoecium results in a horn-like protrusion (arrow) and an exposed ovule (arrowhead). (H) *ett-7* gynoecium has only one reduced ovary (arrows at valve boundaries), with the distal gynoecium appearing Y-shaped from basalized stigmatic and stylar tissue (arrowhead). (I) *ett-7 seu-3* flower showing reduced filamentous sepals and petals (arrowhead and arrow, respectively), few stamens and a stalk-like gynoecium. (J) *ett-7 seu-3* flower showing one normal stamen, one stamen fused along its entire length with the gynoecium (arrowheads) and one valve that is open at the top (arrow). Genotypes described are in Col. Scale bars: 1 mm.

in the first exon, causing a mutation from glutamine to a stop codon. A fourth allele, *seu-4*, was later found in the SALK lines, with a T-DNA inserted into codon 199 (Alonso et al., 2003). *seu-3* and *seu-4* appear to be strong alleles, while *seu-1* and *seu-2* (Franks et al., 2002) are weaker in phenotype.

seu plants have decreased apical dominance and appear shorter overall (Franks et al., 2002). Compared with wild-type flowers, *seu-3* flowers are reduced in size because of smaller petals and stamens (Fig. 1A,B). The most noticeable aspect of *seu-3* flower morphology is the apical cleft in the gynoecium. The stigma and style are split so that two balls of stigmatic tissue are usually seen. Often, the distal valve tips splay out where the style divides in two, resembling small horns. Occasionally these horns have stigmatic papillae at their tips (Fig. 1C). The lack of fusion rarely extends far into the ovary. *seu-3* flowers are semi-fertile and produce few and irregular numbers of seeds when selfed. This is probably due to both male and female fertility defects, as reciprocal outcrosses produce low numbers of seeds (data not shown).

There are significant differences between *seu* alleles in different backgrounds. *seu-3* and *seu-4* are in the Columbia (Col) background, while *seu-1* and *seu-2* are in the Landsberg *erecta* (L*er*) background. Unlike *seu-1* and *seu-2*, *seu-3* does not show homeotic transformations of the outer whorl from sepals to petals, stamens or carpels. Yet when *seu-3* is introgressed into L*er*, it displays more frequent homeotic transformations than does *seu-1* (not shown). In addition, *seu-3* in L*er* has reduced numbers of petals and stamens (like *seu-1*), while *seu-3* in Col has close to wild-type outer whorl organ numbers (Table 1). Finally, fertility of strong *seu* alleles is higher in L*er* than in Col. This may result from greater pollen production and a less substantial split in the gynoecium in L*er*, as measured by the frequency of horn-like valves.

Effect of *seuss* on *ettin* gynoecia

ett flowers have severe gynoecium defects. Apical and basal valve boundaries are displaced towards each other so that overall ovary size is reduced, and sometimes ovaries are completely absent (Fig. 1H). Internal tissue is everted at the apex, revealing internal style and transmitting tract tissue and occasionally exposing ovules.

Flowers of the *ett-7 seu-3* double mutant are markedly different from flowers of either single mutant. Mature flowers are half the size of single mutant flowers (Fig. 1E,I,J). The gynoecium is most often reduced to a stalk-like, ovary-less gynophore topped with stigmatic tissue, although some

Table 1. Effects of *ett-7 seu-3* on organ number and organ shape

Genotype	Number of organs in whorl 1	Number of organs in whorl 2	Number of organs in whorl 3	Number of valves in whorl 4	Percentage of filamentous whorl 1 organs	Percentage of filamentous whorl 2 organs	Number of flowers scored
+/+;+/+	4.0±0.0	4.0±0.0	5.6±0.6	2.0±0.0	0	0	87
ett-7/ett-7;+/+	5.0±0.7	4.6±0.7	5.6±1.1	0.9±0.8	0	1	117
+/+;*seu-3/seu-3*	4.0±0.2	3.8±0.5	5.6±0.6	2.0±0.0	0	<1	156
ett-7/ett-7;*seu-3/seu3*	4.3±1.0	3.9±1.4	3.4±1.3	0.6±0.6	9	73	125

Organs were counted in successive flowers up the inflorescence, beginning with the first formed flower. Fused organs were counted as one organ. The first 30-40 flowers on three or four plants were counted for wild type, *seu-3* and *ett-7*. The first 20-30 flowers on five plants were counted for *ett-7 seu-3*. Gynoecia in whorl 4 were scored as 0, 1 or 2 depending on whether valve tissue was present on none, one or both sides of the gynoecium. All genotypes are in Col.

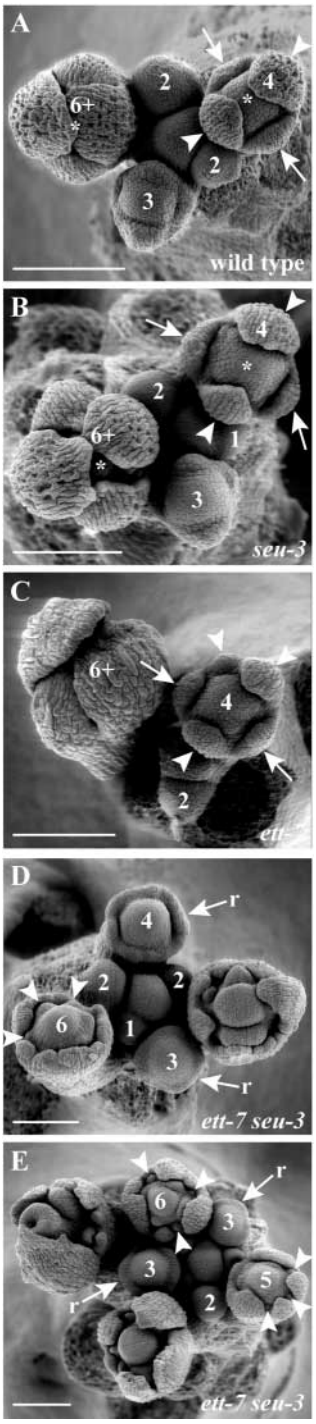

Fig. 2. Effects of *ett-7 seu-3* on floral meristem patterning. Numbers refer to stages of flower development (Smyth et al., 1990). (A) Wild-type inflorescence shows four evenly spaced sepal primordia arising from stage 3 and 4 floral meristems (arrows and arrowheads), and sepals completely enveloping the floral buds by stage 6. (B) Like wild type, *seu-3* has four uniformly positioned sepal primordia (arrows and arrowheads), but sepals are smaller and often do not tightly enclose the inner organs (compare the amount of exposed floral meristem at the asterisks in A and B). (C) *ett-7* flowers characteristically have five sepals, with an extra abaxial sepal (top arrowhead) (D,E) A greater number of developing meristems are present in *ett-7 seu-3* than in either single mutant. *ett-7 seu-3* floral meristems are distinctive by stages 3-4, when an uneven ring of sepal primordia tissue becomes evident (r). As the flower develops, sepals grow out erratically, causing some to be fused and some to be filamentous. Tiny petal primordia in stage 5 and 6 meristems are indicated by arrowheads. Genotypes described are in Col. Scale bars are 0.1 mm.

gynoecia retain a *seu*-like irregular tip (Fig. 1I). There is no eversion of internal transmitting tract or ectopic stigma, as in *ett* single mutants. *ett-7 seu-3* intermittently develops a large ovary (Fig. 1J), which is often split open to reveal malformed ovules. In those few double mutants that develop ovaries, valve length is greater than half the length of the gynoecium 99% of the time, whereas in *ett-7* single mutants, only 16% of the carpels have valves greater than half the length of the gynoecium.

seuss ettin displays severe outer whorl defects

ett affects outer whorl organs such that sepal and petal numbers are increased from four in wild type to an average of five in *ett-7* (Fig. 1D, Table 1). The outer whorls are also affected in *ett-7 seu-3* compared with each single mutant, and this phenotype becomes more severe acropetally. Remaining petals are variably narrowed with most being reduced to mere filaments (Fig. 1E,I). Some sepals are also filamentous, and others are wrinkled and reduced in size (arrowhead in Fig. 1I). Stamens are generally stunted and/or withered. In addition, ~15% of flowers have a stamen fused to the length of the gynoecium (Fig. 1J). Organ numbers are more variable in whorls one and two, and reduced in whorl 3 (Table 1) compared with organ numbers in *ett-7* or *seu-3* single mutants. Phyllotaxy in the outer two whorls is disrupted and organ spacing becomes increasingly disorganized in later arising flowers, so that it becomes difficult to distinguish whorl 1 from whorl 2. A few sepals and petals are recognizable, but most organs have aberrant morphologies. These organs sometimes appear to develop from the same (merged) outer organ whorl.

Disparities in *ett-7 seu-3* floral organ position, shape and size can be seen at primordia inception (Fig. 2). In wild type, sepals originate from the floral meristem as four distinct bulges, which overlie the meristem by stage 6 (Fig. 2A). *seu-3* sepals develop similarly, with initiation of four discrete sepal primordia occurring at stage 3 (Fig. 2B). The sepals in the lateral plane arise slightly later than the sepals in the medial plane and are therefore smaller (arrows versus arrowheads in Fig. 2A,B). Beginning at stage 4, a modest difference can be seen between *seu-3* and wild-type flower development. *seu-3* sepals appear smaller and often do not completely enclose the bud, leaving some of the developing meristem exposed (compare asterisks in Fig. 2A with 2B). In *ett-7*, although there are five sepal primordia, they are clearly distinct from each other as with wild-type sepals (Fig. 2C).

Sepal initiation in the *ett-7 seu-3* double mutant is noticeably different than in either single mutant. Sepals arise as one uneven ring of tissue, with no distinct boundaries between individual primordia (Fig. 2D,E). Observation of sepal primordia at stages 5-6 often provide an inflated impression of sepal number, because some of these primordia cease to develop and others appear congenitally fused in their basal halves. The ring of sepal tissue eventually grows out erratically to produce some mature organs. Petal and stamen primordia

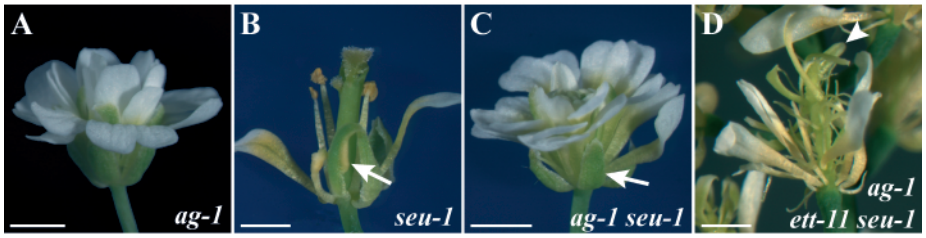

Fig. 3. Flowers of *ag-1* (A), *seu-1* (B), *ag-1 seu-1* (C) and *ag-1 ett-11 seu-1* (D). Stamenoid sepals in *seu-1* (arrow in B) do not occur in *ag-1 seu-1* (arrow in C). Filamentous organs in *ett seu* are also present in *ag-1 ett-11 seu-1* (compare Fig. 1I with D). The floral meristem in *ag-1 ett-11 seu-1* continues to grow but occasionally stops producing lateral organs (arrowhead in D). Genotypes described are in L*er*. Scale bars: 1 mm.

are difficult to identify because of their aberrant positioning. Some stamen primordia appear incompletely separated from the meristem proper (not visible in Fig. 2), which may portend the high frequency of mature stamens fused with the gynoecium. Together, these observations demonstrate that *seu-3* and *ett-7* together affect morphology and positioning of floral organs, including sepal partitioning, petal shape and stamen number.

Effect of *agamous* on *ettin seuss*

seu causes ectopic and precocious *AG* expression in whorls one and two, leading to partial homeotic transformations of sepals into mosaic carpelloid, stamenoid or petalloid organs (Franks et al., 2002). AG is a MADS-box transcription factor responsible for stamen and carpel identity, as well as for determinacy of the floral meristem (Bowman et al., 1989; Yanofsky et al., 1990). This latter growth suppression role is supported not only by the indeterminate flower in the *ag* mutant, but also by evidence that ectopic expression of *AG* in whorl 2 under the *AP3* promoter inhibits growth of whorl 2 petals (Jack et al., 1997). If *AG* expression suppresses petal growth and affects floral organ identity, the ectopic *AG* present in *seu* could be responsible for the filamentous organ phenotype in the *ett seu* double mutant.

The null *ag-1* allele produces flowers with petals and sepals in place of stamens and carpels (Fig. 3A). In addition, the indeterminate floral meristem produces an indefinite number of additional organ whorls as (sepal, petal, petal)^n. The *seu-1* mutant in L*er* occasionally has a mosaic sepal, owing to the ectopic expression of *AG* (Fig. 3B). When *ag-1* is crossed with *seu-1*, AG protein activity is removed and sepals no longer exhibit homeotic transformations (Franks et al., 2002). An *ag-1 seu-1* double mutant retains the organ identity and indeterminacy of *ag-1* single mutant flowers, causing reiterating whorls of sepals, petals, petals (Fig. 3C). An *ag-1 ett* double mutant also resembles *ag-1* in its floral organ identity and floral meristem indeterminacy (Sessions et al., 1997). Like the double mutants, an *ag-1 ett-11 seu-1* triple mutant produces a repeating pattern of sepals, petals, petals (Fig. 3D). However, both sepals and petals are variably narrowed, reminiscent of the *ett seu* double mutant phenotype. Each flower contains recognizable sepals and petals in its initial whorls, but as the floral meristem develops acropetally, the organs become increasingly filamentous. Floral meristems occasionally continue to elongate without producing lateral organs (Fig. 3D, arrowhead). Determination of organ numbers in these *ag ett seu* mutants is difficult because organs are not evenly spaced around the floral meristem and individual whorls cannot be distinguished from each other. Nevertheless, it is clear that removing ectopic and precocious AG from *ett seu* by generating *ag ett seu* does not rescue the filamentous, mispositioned organ phenotype present in the *ett seu* double mutant.

Classic auxin responses are decreased in *seuss*

Owing to the synergistic phenotype between *seu-3* and *ett-7*, we reasoned that *SEU*, like *ETT*, may have a role in auxin response. In support, *seu* plants are shorter with increased shoot branching, and after 5 weeks *seu-3* develops an average of six lateral inflorescences from the rosette, instead of the three lateral inflorescences typically found in wild type (Fig. 4A). Such decreased apical dominance is commonly found in auxin resistant mutants such as *axr1* (Lincoln et al., 1990). Because many auxin signaling mutants also have root defects, *Arabidopsis* roots have become a well-characterized system with which to study auxin signaling. To determine whether *seu-3* affects auxin response, we analyzed *seu*-3 roots for three archetypal auxin responses: lateral root initiation, resistance to exogenous auxin and gravitropic response.

seu-3 possesses fewer lateral roots than does wild type (Fig. 4B). Some *seu-3* seedlings have no lateral roots, while most *seu-3* seedlings have half of the number found in wild-type roots (Fig. 4B). Close examination of these *seu-3* roots indicates fewer points of lateral root initiation from the pericycle, resulting in fewer mature lateral roots. When exogenous auxin in the form of naphthaleneacetic acid (NAA) is added to growth medium, primary root elongation is inhibited in wild-type plants. Cotyledons also grow downward (epinasty) owing to differential elongation of adaxial and abaxial surfaces. In the presence of NAA, *seu-3* roots are inhibited less and therefore elongate more than wild-type roots (Fig. 4C,D). Moreover, *seu-3* cotyledons are not epinastic like those of wild type (Fig. 4E,F). Therefore, *seu-3* is less sensitive to the inhibitory effects of NAA. To determine the effect of the *seu-3* mutation on root gravitropsim, root tip angles were measured 24 hours after rotating the vertically grown seedlings 90°. While wild-type roots are consistently aligned to the gravity vector, *seu-3* roots are less likely to orient properly (Fig. 4G). These phenotypes – decreased apical dominance, fewer lateral roots, reduced sensitivity to exogenous auxin in both the root and the shoot, and more variable growth vector in the root – suggest that *seu* has a decreased response to auxin.

Expression of the DR5 auxin response reporter is reduced in *seuss*

To further test the hypothesis that *seu* displays diminished auxin response, we examined expression of the synthetic auxin response reporter DR5::GUS (Ulmasov et al., 1997). *seu-3*

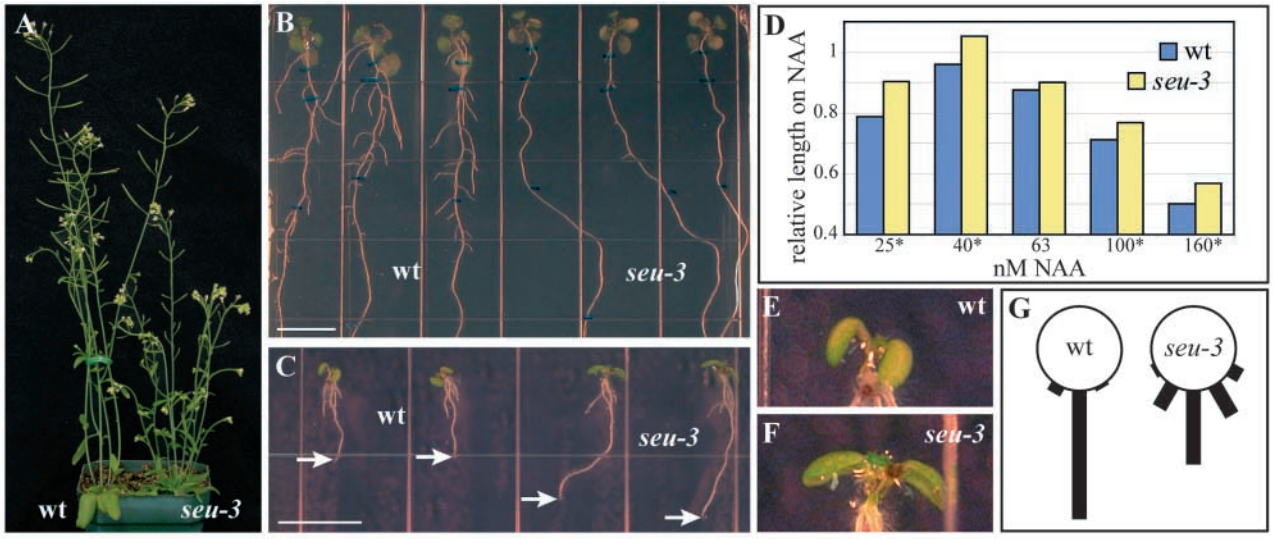

Fig. 4. *seu-3* displays hallmark auxin response phenotypes. (A) *seu-3* is shorter than wild type with increased shoot branching. (B) The three *seu-3* seedlings (right) produce fewer lateral roots than the three wild-type seedlings (left). (C) *seu-3* roots are longer than wild-type roots when grown on media containing 100 nM NAA. This indicates that *seu-3* is not as sensitive to the inhibitory effects of exogenous auxin on root elongation. (D) Quantification of differences in primary root length in seedlings grown on media lacking hormone relative to seedlings grown on media containing NAA ($n \geq 24$). *$P<0.01$ (*t*-test). (E,F) Close-up of seedling apices from C. Compare epinastic cotyledons of wild type (E) with more upright cotyledons of *seu-3* (F). (G) Angle of root tips in light grown seedlings, 24 hours after rotating the plates 90°. Length of each line represents the number of seedlings with root tips growing at that angle, in 12 30° categories (n=52). *seu-3* roots reorient to the gravity vector less successfully than do wild-type roots [$P<1*10^{-4}$ (*t*-test)]. Genotypes described are in Col. Scale bars: 1 cm.

shows a clear decrease in DR5 expression in both the root and the shoot. In wild type, DR5 expression in the root meristem extends into the root cap and stele, whereas *seu-3* roots have decreased DR5 expression in the root cap and no expression in the developing vascular tissue (Fig. 5A,B). The attenuation of DR5 expression in *seu-3* is even more apparent in the shoot. DR5 expression in 5-day-old wild-type seedlings is most intense at the distal leaf tip, where auxin is produced, and also is present throughout the leaf in incipient secondary and tertiary veins (Fig. 5C). DR5 expression in *seu-3* seedlings, however, occurs only occasionally at the leaf tip and in hydathodes (Fig. 5D). In 7-day-old wild-type seedlings, DR5 expression occurs in the basal half of the leaf, paralleling the basipetal differentiation of vascular tissue (Fig. 5E). *seu-3* seedlings never show DR5 expression in these procambial sites of future vein formation (Fig. 5F). That sites of maximum auxin response in both the root and the leaves are diminished in *seu-3* supports a role for *SEU* in auxin signaling.

Floral organ formation in *seuss pinoid* is severely compromised

The serine threonine kinase PINOID (PID) has been postulated to affect polar auxin transport as well as auxin signaling (Benjamins et al., 2001; Christensen et al., 2000). Because the pleiotropic effects of *pid* on flower development are presumed to be auxin related, we examined *pid seu* double mutants for effects of *seu* on the *pid* floral phenotype. *pid* inflorescences produce only a few flowers before terminating in a pin-like structure reminiscent of the *pin1* auxin transport mutant (Bennett et al., 1995; Okada et al., 1991). *pid* flowers have larger, supernumerary petals but fewer sepals and stamens. Organs within each whorl are often fused together, frequently resulting in heart-shaped petals (Fig. 6A). The central whorl of *pid* flowers phenocopies *ett* gynoecia in that valve length is variably reduced, decreasing the size of the ovaries (Fig. 6B).

pid-1 seu-3 double mutants exhibit a strikingly synergistic phenotype. Very few outer whorl organs develop in *pid-1 seu-3* flowers, resulting in clusters of naked gynoecia (Fig. 6C,D). While developing gynoecia are normally enveloped by sepals in wild-type flowers, *pid-1 seu-3* flowers rarely contain a wrinkled sepal, a filamentous petal or stunted stamens (Fig. 6C-F). More often the remnants of the floral meristem appear as a swollen ring at the base of the gynoecium, without evidence of distinct primordia (Fig. 6C,D). Although half-formed sepals occasionally develop from this region, there is no discernible boundary between the swollen area and the pedicel (Fig. 6D). Lack of pedicel elongation may contribute to that region becoming overly bulbous. In fact, double mutant plants are shorter and internode length is reduced compared with those features in either single mutant (not shown).

The *pid-1 seu-3* double mutant gynoecium displays characteristics of both *pid-1* and *seu-3* mutants. Like *pid-1*, the central whorl of *pid-1 seu-3* most often develops as a long gynophore topped with stigmatic tissue (Fig. 6E). The gynoecium is frequently missing major tissues, such as valves, and often is split into multiple stalks, as if carpel fusion did not occur normally (Fig. 6F).

Evaluation of organ numbers confirms the strong interaction between *pid-1* and *seu*-3 (Fig. 7). Wild-type L*er* flowers typically produce four sepals, four petals, six stamens and two carpels. When *seu-3* is in the L*er* background, petal and stamen numbers are decreased slightly, with a mean of 3.1 petals and 5.0 stamens. Sepal and carpel numbers in *seu-3* are similar to wild type. In *pid-1*, variability in organ number increases, with

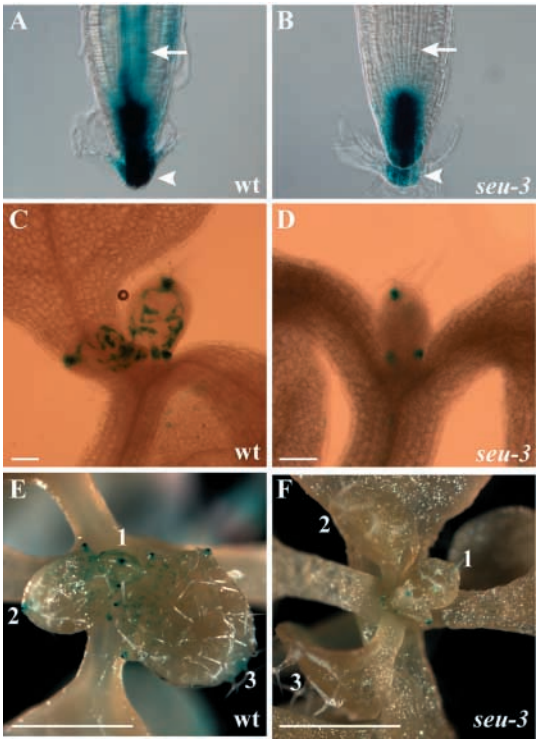

Fig. 5. Seedlings expressing the auxin response reporter DR5::GUS. DR5 is expressed more strongly in wild type (A,C,E) than in *seu-3* (B,D,F). (A) Roots assayed for GUS activity show GUS staining extending from the root meristem into the root cap (arrowhead) and stele (arrow) in wild type. (B) *seu-3* roots display a substantial decrease in GUS product, with only weak DR5 expression in the root cap (arrowhead), and none in the vasculature (arrow). (C,D) Five-day-old seedlings show first leaves with more extensive GUS activity in wild type, spatially marking incipient and developing veins, whereas in *seu-3* GUS product is seen only at the distal leaf tip and in hydathodes. (E,F) Seven-day-old *seu-3* seedlings do not show a recovery in GUS activity. As leaves grow and mature, wild-type GUS activity parallels the basipetal differentiation of vascular tissue. No GUS product is seen in *seu-3* leaves at similar stages (compare 1, 2 and 3). Genotypes described are in Col.

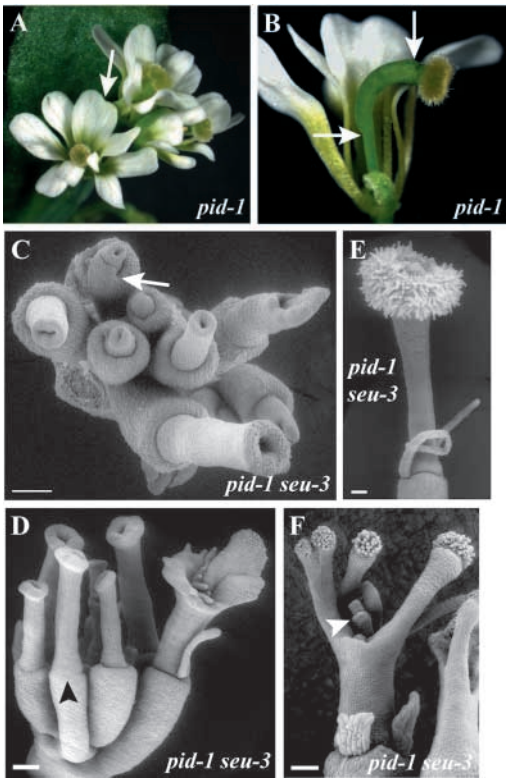

Fig. 6. Effect of *seu-3* on the auxin regulatory mutant *pid-1*. (A) *pid-1* inflorescence showing flowers with large, supernumerary petals that are occasionally fused (arrow). (B) *pid-1* flower showing gynoecium with an infrequent ovary that, like *ett* ovaries, is reduced in size (arrows indicate top and bottom of single valve). (C) Top-down view of *pid-1 seu-3* inflorescence showing naked gynoecia. A swollen ring of tissue appears in place of the outer whorl organs. Rarely, a sepal originates from this band of tissue (arrow). (D) Side view of *pid-1 seu-3* inflorescence showing that the swollen tissue in C is continuous with the pedicel. Occasionally there is a less defined boundary between the pedicel and the gynoecium (arrowhead). (E) *pid-1 seu-3* gynoecium that resembles *pid-1* single mutant gynoecium, a stalked organ without valves. Two filamentous outer whorl organs are apparent. (F) *pid-1 seu-3* gynoecium that is split into multiple stigma-capped stalks. Although no valves have differentiated, the gynoecium still produces ovules (arrowhead). Genotypes described are in L*er*.

a median of three sepals, six petals, three stamens and no valves. The double mutant produces a mean of 0.5 sepals, 0.5 petals, 0.5 stamens and 0.1 valves, with median numbers of zero for each organ type. Those few flowers that generate organs tend to be the first few in an inflorescence, with the lack of organ development increasing in later-formed flowers. This drastic reduction in organ numbers in *pid-1 seu-3* suggests a synergistic interaction between these two mutants, especially in whorl two, where the increased number of petals in *pid-1* is reduced to almost none in *pid-1 seu-3*.

SEUSS and ETTIN physically interact

SEU is postulated to be a transcriptional co-regulator based on (1) its repression of *AG* in the outer whorls of the floral meristem, and (2) its sequence similarity with transcriptional co-factors such as Ldb1 in mouse and Chip in *Drosophila* (Franks et al., 2002). Given the identity of SEU as a putative transcriptional co-regulator and ETT as an ARF transcription factor, we tested whether SEU and ETT directly interact. Results from a yeast two hybrid assay suggest that SEU and ETT physically interact (Fig. 8). When ETT is expressed in a bait vector (ETT-BD) and SEU is expressed in a prey vector (SEU-AD), yeast grow robustly with uracil selection for the interaction. Yeast do not grow with ETT-BD and an empty prey vector (AD), or with SEU-AD and an empty bait vector (BD). SEU-AD does not interact non-specifically with all prey, as shown by lack of growth of yeast containing SEU-AD and UFO-BD.

To determine whether the interaction between SEU and ETT is specific to ETT/ARF3 among ARFs, growth of yeast expressing SEU-AD and ARF1 in a bait vector (ARF1-BD) was also examined. Yeast expressing ARF1-BD and SEU-AD show no growth at day 3, similar to ARF1-BD and AD. Thus, SEU interacts with ETT, but not with ARF1 in a yeast two hybrid system.

Discussion

This work describes new alleles of the *seu* mutant along with morphological, physiological and genetic evidence of a role for *SEU* in auxin-regulated growth and development. *seu* displays several classic auxin response defects, such as reduced apical dominance, decreased lateral root initiation, resistance to exogenous auxin and diminished expression of the auxin response reporter DR5. This collectively suggests that *SEU* functions in the auxin signaling pathway. *seu* interacts synergistically with the auxin response pathway mutants *ett* and *pid*. In addition, SEU interacts molecularly with the auxin response factor ETT/ARF3, which suggests that SEU may regulate transcription of auxin response genes in concert with ETT.

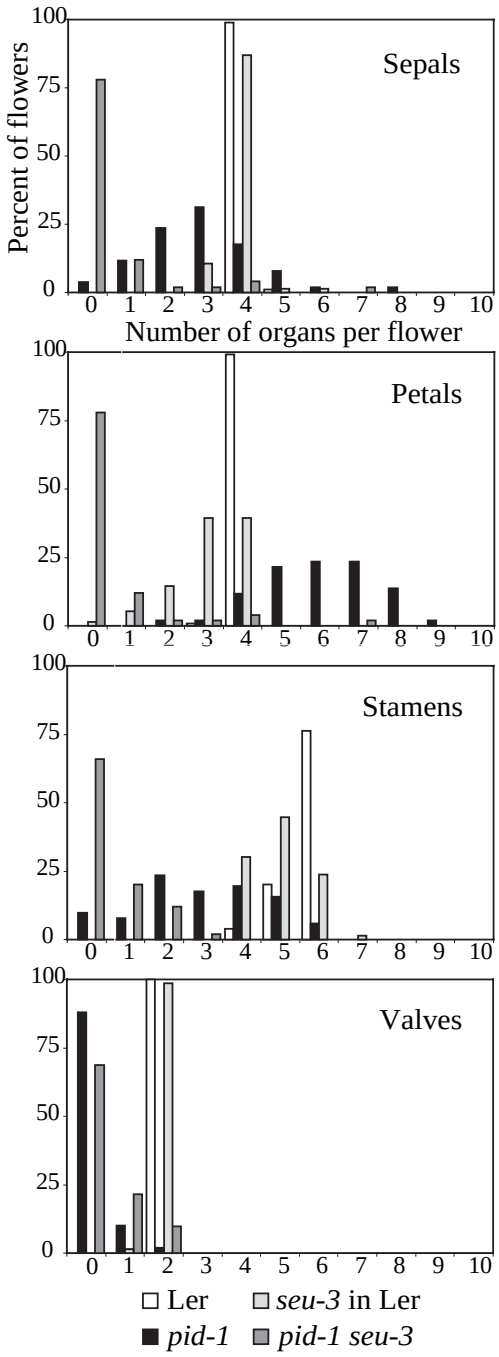

Fig. 7. Distribution of floral organ numbers in 105 wild-type flowers, 51 *pid-1* flowers, 76 *seu-3* flowers and 50 *pid-1 seu-3* flowers. Organs fused together were counted as one organ. Gynoecia in whorl 4 were scored as 0, 1 or 2 depending on whether valve tissue was present on none, one or both sides of the gynoecium. *pid-1 seu-3* flowers have very few outer whorl organs. All genotypes are in L*er*.

SEUSS functions with *ETTIN* to promote floral organ development

seu-3 was originally identified as a modifier of the *ett* floral mutant. *ETT* affects multiple patterning events during flower development, including number and spacing of perianth organs and regional differentiation of reproductive organs (Sessions et al., 1997). *ETT* function has been best characterized in the gynoecium because *ett* ovaries are severely reduced in size, with the missing ovary tissue replaced by abaxialized style and transmitting tract tissues. In the *ett seu* double mutant, the few ovaries that are produced are longer, making *ett* appear suppressed in the central whorl. *SEU* promotes development of marginal tissue between the valves that gives rise to style, transmitting tract and ovules (R. G. Franks, personal communication). The partial suppression of *ett* may be explained by the lack of development of these tissues in *ett-7 seu-3*, which normally overproliferate in *ett*.

In the outer whorl organs, the interaction of *seu* and *ett* appears synergistic. In *seu*, sepals and petals are narrower and stamens produce little pollen. In *ett*, there is a slight increase in number of sepals and petals, affecting the spacing between organs. The *ett seu* double mutant exhibits a more severe phenotype, with substantial decrease in stamen number and filamentous mispositioned perianth organs. The spacing between organs is disrupted not only within whorls but between whorls, so that stamens are occasionally fused with the gynoecium. This suggests that *SEU* and *ETT* participate in convergent pathways in the floral meristem for positioning and growth of floral organs. It is not known whether *SEU* and *ETT* expression patterns overlap. By northern analysis, *SEU* is expressed at low levels in all tissues analyzed (Franks et al., 2002). *ETT* is expressed in incipient floral meristems, but later expression is restricted to vascular bundles of petals and stamens and the abaxial side of the gynoecium where valves and replum differentiate (Sessions et al., 1997).

The *SEUSS ETTIN* pathway is distinct from the *SEUSS AGAMOUS* pathway

Double mutant analysis of *ett* with homeotic mutants involved in the ABC model of floral organ development established that *ETT* does not play a role in floral organ identity (Sessions et al., 1997). By contrast, *SEU* causes ectopic and precocious expression of the MADS box transcription factor *AGAMOUS*, responsible for stamen and carpel identity (Franks et al., 2002). Removing this misexpressed AG from the outer floral whorls by generation of an *ag-1 ett-11 seu-1* triple mutant does not affect the filamentous, mispositioned organ phenotype caused by *ett* and *seu*. Thus, the effects of *SEU* on *ETT* are independent of the repression of *AG* by *SEU*. These experiments suggest that *SEU* and *ETT* act in a distinct pathway affecting floral organ positioning and development.

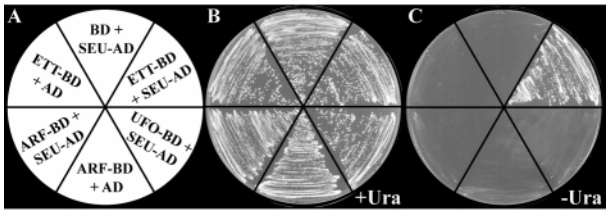

Fig. 8. Yeast two hybrid assay. (A) Schematic of bait and prey combinations. (B) Growth of yeast on selection for bait and prey plasmids only (+Ura). (C) Growth of yeast on selection (uracil biosynthesis) for bait and prey interaction (–Ura). BD, empty vector pBD; AD, empty vector pGAD424; SEU-AD, SEU in pGAD424; ETT-BD, ETT in pBD; UFO-BD, UNUSUAL FLORAL ORGANS in pAS1; ARF-BD1, ARF1 in pGBT9.

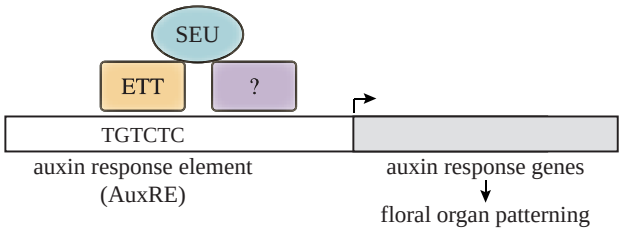

Fig. 9. Experimental model of SEU function. The SEU transcriptional co-regulator controls floral meristem patterning in response to auxin in conjunction with ETT, an ARF transcription factor. ETT binds to AuxREs in the promoters of auxin responsive genes. SEU interacts with ETT and other regulatory factors to affect transcription of those targets.

SEUSS is a novel factor affecting auxin response

Several facets of the *seu* phenotype resemble hallmark defects of auxin response. Auxin is required in the root for organization of the meristem, gravitropic response, primary root elongation and initiation of lateral roots (Sabatini et al., 1999; Moore, 2002; Casimiro et al., 2003). The meristem in *seu-3* roots appears to function normally, but after reorientation of vertically grown seedlings, the angle of root growth in *seu-3* is more variable than in wild type. *seu-3* produces half as many lateral roots as wild type, suggesting that either not enough auxin is present in the root zone where lateral roots initiate, or that the auxin present is not perceived as efficiently as in wild type.

To distinguish between these hypotheses and to confirm that *seu* affects auxin response, *seu-3* seedlings were grown in the presence of exogenous NAA, a biologically active auxin. *seu-3* roots are longer relative to wild-type roots when grown on NAA, indicating that *seu-3* root growth is less inhibited by exogenous auxin than wild-type roots. That *seu-3* is less responsive to NAA treatment than wild type indicates *seu* is defective in auxin perception or response.

Further insight into the role of *SEU* in auxin response was gained by examining expression of the auxin response reporter DR5. If *seu* is unable to transport auxin normally, auxin would pool at sites of synthesis and be depleted in other tissues. This perturbed auxin flux would be reflected in an increase in DR5 expression at the distal leaf tip and a decrease in expression in outlying areas of vascular development. However, in *seu*, DR5 expression is reduced but not displaced in both developing leaves and the root tip, supporting the hypothesis that *seu* is defective in perception of/response to auxin within a cell and not in transport of auxin between cells.

seuss flowers are sensitized to disruptions in auxin flux

The striking abolishment of outer whorl organs in the *pid-1 seu-3* double mutant demonstrates a strong synergism between *seu-3* and *pid-1*. Because *pid* is thought to destabilize auxin levels in the inflorescence meristem, the *seu-3 pid-1* interaction further implicates *SEU* in floral auxin response. Based on ectopic expression data, the PID kinase has been postulated to both positively regulate auxin transport and negatively regulate auxin signaling (Benjamins et al., 2001; Christensen et al., 2000). PID probably affects regional auxin distribution in the inflorescence meristem, because the auxin efflux carrier PIN1 is reduced and mislocalized in developing *pid* meristems (Reinhardt et al., 2003). This disruption in auxin flux, together with the decreased auxin response of *seu*, may account for the lack of organ primordia development in *pid-1 seu-3* double mutant flowers. Those few outer whorl organs that develop in *pid-1 seu-3* are stunted and/or filamentous, similar to organs in *ett-7 seu-3*. The parallel organ phenotypes in these double mutants supports the hypothesis that *SEU* functions in auxin response, with *pid-1* compromising *seu* floral meristems even more severely than *ett*, owing to reduced auxin levels as well as reduced auxin response. Transient application of the polar auxin transport inhibitor NPA to wild-type inflorescences also reduces the number of outer whorl floral organs that develop in affected flowers (Nemhauser et al., 2000). By contrast, micro-application of the natural auxin indole-3-acetic acid (IAA) promotes lateral outgrowth of both leaves and flowers (Reinhardt et al., 2000). The rings of meristem tissue that develop in both *ett-7 seu-3* and *pid-1 seu-3* bear similarity to the rings of flowers and floral organs that develop in the auxin transport mutant *pin1* after treatment with IAA at the top of the *pin1* inflorescence meristem (Reinhardt et al., 2000). Taken together, the above findings suggest that lack of organ development in *pid-1 seu-3* is due to insufficient levels of auxin and deficient response to auxin within organ primordia.

The severity of the *pid-1 seu-3* phenotype suggests several possible roles for the PID kinase in the *ETT-SEU* pathway. Because *PID* has an AuxRE in its promoter and is auxin-inducible (Benjamins et al., 2001), SEU and ETT may function together to directly affect *PID* transcription. A second possibility is that *PID* indirectly affects the *SEU* pathway by its effect on auxin transport and regional auxin levels (Benjamins et al., 2001; Reinhardt et al., 2003).

A model for *SEUSS* action

The collective auxin response phenotypes of *seu* may reflect a novel and fundamental role for *SEU* in auxin-mediated signal transduction. Based on the genetic and physical interactions of *SEU* with *ETT* (Figs 1, 2, 8), we propose that SEU functions with ETT to regulate transcription of auxin response genes involved in floral meristem patterning (Fig. 9).

How might a SEU-ETT transcriptional complex regulate gene transcription? SEU contains two remarkably glutamine-rich domains that may function as transcriptional activation patches (Mitchell and Tjian, 1989; Petcherski and Kimble,

2000). However, *SEU* represses *AG* (Franks et al., 2002), suggesting that SEU may mediate activation and/or repression of target genes, depending on the cellular context (Freiman and Tjian, 2002; Mannervik et al., 1999). ETT is thought to function as a repressor based on protoplast transfection assays (Tiwari et al., 2003; Ulmasov et al., 1999b) and ectopic expression of the *SPATULA* (*SPT*) transcription factor in whorl 4 primordia of the *ett* mutant (Heisler et al., 2001). Moreover, ETT lacks the highly conserved ARF domains III and IV that are responsible for dimerization of ARFs and Aux/IAAs, which regulates ARF activity (Dharmasiri et al., 2002). The absence of domains III and IV in ETT, and the distinct C-terminal region of ETT, suggest that ETT acts differently than other ARFs. SEU may contribute to and/or regulate this unique function of ETT.

SEU is homologous in its central region with mouse Ldb1, which homodimerizes and binds LIM-homeodomain transcription factors to form a tetrameric complex (Jurata et al., 1998). SEU may similarly act as a bridging factor between ETT and other regulatory molecules (Fig. 9). These regulatory molecules could include ARFs and/or Aux/IAAs, which would allow regulation of the ETT transcriptional complex by the Aux/IAA family. However, SEU interacts with ETT/ARF3 but not with ARF1, another putative ARF repressor, in a yeast two-hybrid assay (Fig. 8). These results suggest a testable hypothesis that SEU interacts specifically with ETT among ARFs. In this case, ETT and SEU may function together in regions outside the flower, as *seu* mutants have global auxin response defects, and both *SEU* and *ETT* are expressed in a variety of plant tissues (Franks et al., 2002; Sessions et al., 1997; Ulmasov et al., 1999b). The present research demonstrates a role for *SEU* in auxin-mediated floral organ development. Future studies will clarify the role of *SEU* in transcriptional regulation of auxin response genes, and will undoubtedly reveal additional novel players in this complex developmental program.

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