The role of *JAGGED* in shaping lateral organs

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Summary

Position-dependent regulation of growth is important for shaping organs in multicellular organisms. We have characterized the role of *JAGGED*, a gene that encodes a protein with a single C_2H_2 zinc-finger domain, in controlling the morphogenesis of lateral organs in *Arabidopsis thaliana*. Loss of *JAGGED* function causes organs to have serrated margins. In leaves, the blade region is most severely affected. In sepals, petals and stamens, the strongest defects are seen in the distal regions. By monitoring cell-cycle activity in developing petals with the expression of *HISTONE 4*, we show that *JAGGED* suppresses the premature differentiation of tissues, which is necessary for the formation of the distal region. The localization of defects overlaps with the expression domain of *JAGGED*, which is restricted to the growing regions of lateral organs. *JAGGED* expression is notably absent from the cryptic bract, the remnant of a leaf-like organ that subtends the flower in many species but does not normally develop in wild-type *Arabidopsis*. If misexpressed, *JAGGED* can induce the formation of bracts, suggesting that the exclusion of *JAGGED* from the cryptic bract is a cause of bractless flowers in *Arabidopsis*.

Supplemental data available online

Key words: *Arabidopsis thaliana*, Leaf development, Bract development, Flower development, *JAGGED*, *APETALA1*

Introduction

The aerial part of the plant develops new lateral organs continuously through a reiterative process of organogenesis that takes place at the shoot apical meristem. During the life cycle, these lateral organs are modified to suit changing needs. In flowering plants, lateral organs will take the shape of leaves, sepals, petals, stamens and carpels. Leaves typically develop a wide blade adapted to gather light, whereas floral organs promote reproduction. Throughout evolution, lateral organs have also been modified to help plants adapt to almost every terrestrial habitat on earth. For example, the leaves of cactus plants have been modified to form spines to protect against herbivores.

While our understanding of the specification of organ identity is advanced, a mechanistic understanding of the processes that sculpt lateral organs is only now emerging. In *Antirrhinum*, two members of the TCP family of transcription factors, encoded by *CINCINNATA* (*CIN*) and *CYCLOIDEA* (*CYC*), have been implicated in the regulation of lateral organ development (Cubas et al., 1999). *CIN* controls growth of the leaf blade and is required to generate flat leaves with zero Gaussian curvature (Nath et al., 2003). *cin* mutants develop excessive growth at the periphery of the leaf blade, resulting in wavy leaves. Interestingly, *CIN* appears to prevent excessive growth by sensitizing peripheral cells to a cell-cycle arrest front that moves from the tip of the leaf to the base. *CYC*, which also appears to regulate growth via effects on the cell cycle, suppresses growth of dorsal floral structures, producing the asymmetric flowers typical of *Antirrhinum* (Gaudin et al., 2000; Luo et al., 1996). It is interesting to note that both genes appear to affect organ shape by promoting differentiation.

We have characterized *JAGGED* (*JAG*) (formerly *BRACTS*), a gene involved in the formation of lateral organs. *JAG* encodes a putative transcription factor with a single C_2H_2 zinc-finger domain and is expressed in the growing regions of lateral organs. Mutations in *JAG* most severely affect the distal regions of organs, resulting in a jagged edge. In petals, the entire distal half of the organ can be eliminated. In *jag* mutants, cell-cycle activity declines earlier that in wild type, suggesting that *JAG* promotes distal petal development by suppressing premature cell-cycle arrest. Misexpression studies with an activation-tagged allele reveal that *JAG* can promote the growth of many different tissues including the cryptic bract. We discuss a possible role for *JAG* in maintaining growth of lateral organs and in the diversification of plant form.

Materials and methods

Definition of bracts

Terminologies to differentiate between the leaf types of a flowering plant are varied. In loose definitions, a bract is a flower- or inflorescence-associated leaf, without a distinction between inflorescence-associated leaves in general and ones that specifically subtend a flower (Harris and Harris, 1994). We adopt the terminology of Irish and Sussex (Irish and Sussex, 1990) and Bowman and

colleagues (Bowman et al., 1993), who describe a bract as being elongate, with a pointed tip, lacking a petiole (leaf stalk), having stipules and being delayed in senescence compared to sepals. We do not consider epidermal cell pattern in the classification of bracts, since there can be differences between bracts produced early and late in development. Bracts are similar to cauline leaves, which develop on the inflorescence. Cauline leaves that are sessile and subtend co-florescence shoots, can be considered bracts sensu lato. They are distinguished from rosette leaves, which develop during the vegetative phase of growth and have a defined petiole at their base (Bowman, 1993).

Plant growth and material

Plants were grown under a 3:1 ratio of Cool White and Gro-Lux (wide spectrum) fluorescent lights at 23°C in long day conditions (16 hour light, 8 hour dark).

Wild type was either Columbia [Col-0, Col *glabrous* (Col (*gl*)] or Wassilewskija (Ws). *jag-1* and *jag-4* were identified by screening the University of Wisconsin Knockout collection (Krysan et al., 1999) using *JAG*-specific primers N-1365 and N-1366 (see Supplementary Table 1 for oligonucleotide sequences; Table S1, http:// dev.biologists.org/supplemental). The *jag-1* allele is disrupted by a T-DNA derived from pSKI015; *jag-4* by a T-DNA derived from pD991, which contains an *AP3::GUS* fusion. As has been observed in other lines derived from this collection (http:// www.biotech.wisc.edu/Arabidopsis/), *jag-4* has defects in the development of petals and stamens that appear to be due to co-suppression by *AP3* sequences in the T-DNA (see Fig. S1, http://dev.biologists.org/supplemental). The *jag-5D* mutation was obtained from a population of Col(*gl*) *pop1*$^{-/-}$ plants (Preuss et al., 1993) transformed with pSKI015 (Weigel et al., 2000). *jag-5D* is linked to the *pop1* mutation. The effect of *jag-5D* on lateral organ development was the same in Col-0 and Col(*gl*). *ap1-15* is in Col-0 (Ng and Yanofsky, 2001). Transgenic Col-0 plants were generated by floral-dip method (Clough and Bent, 1998).

Genotyping of *jag-1* used primers oJD126 and oJD127, to amplify a 0.7 kb fragment from the wild-type locus. To detect the *jag-1* allele, a T-DNA-specific oligonucleotide, JL202, and N-0681 were used to amplify a 1.1 kb fragment. Genotyping of *jag-5D* was done using N-1509, N-1510 and SKC12 in a single PCR reaction. PCR products were digested with *Eco*RV producing a band of 0.5 kb representing the wild-type allele and a band of 0.4 kb representing *jag-5D*. *jag-5D* homozygotes can also be distinguished by phenotypes associated with the *pop1* mutation (Preuss et al., 1993). Genotyping of *ap1-15* was as described previously (Ng and Yanofsky, 2001).

Biometric analysis

The third and fourth rosette leaves from Ws and *jag-1* plants were scanned. Area and perimeter of leaf blade region were measured using the NIH Image program (developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

Cloning of *JAG* genomic locus and cDNA

Plasmid rescue of *jag-5D* genomic DNA was performed as described previously (Weigel et al., 2000). pJDP2, which was used for *jag-5D* recapitulation, was plasmid rescued from *Pst*I digested *jag-5D* genomic DNA. An initial *JAG* cDNA clone was obtained by RT-PCR (see below) using primers targeting exonic sequences predicted by Genscan (Burge and Karlin, 1997). 5′ and 3′ RACE (rapid amplification of cDNA ends) was performed using the First Choice RLM-RACE kit (Ambion, Inc.) according to the manufacturer's instructions. A full-length cDNA was obtained using N-0335 and N-0336. An initial *JAGGED-LIKE* (*JGL*) cDNA was amplified based on the At1g13400 gene model. A full-length *JGL* clone was obtained using oJD119 and oJD120 (pPY1).

Construction of transgenic lines

The *JAG* recapitulation construct pJD71 was made by isolating a *Hin*dIII/*Cla*I fragment containing the genomic region and *35S* enhancer elements from pJDP2 and subcloning it into pBJ36 (pJD63) (Gleave, 1992). The cassette was then shuttled into pART27 (pJD71). For *AP1::JAG*, the *JAG* cDNA was PCR amplified from first-strand cDNA with *Pfu* Turbo polymerase (Stratagene), using primers N-0680 and N-0681, to introduce *Hin*dIII and *Bam*HI restriction sites. The product was cloned into pBluescript-SK$^+$ and sequenced (pJD37). The *JAG* fragment was subcloned into pJD33 (Wu et al., 2003), replacing the *AP1* cDNA (pJD41). For *AP1::JAG:GFP*, the *JAG* cDNA was PCR amplified from pJD37 with N-0680 and N-1413, adding a *Hin*dIII site to the 5′ UTR and replacing the stop codon with a *Pst*I site (pJD62). *GFP* coding sequence was amplified from pCAMBIA1302 (Hajdukiewicz et al., 1994), replacing the start codon with a *Pst*I site and adding an *Xba*I site to the 3′ UTR (pJD60). *JAG* was then translationally fused to *GFP* and shuttled into pJD51 (Wu et al., 2003), replacing *AP1:GFP* with *JAG:GFP* (pJD105).

Scanning electron microscopy

Samples for scanning electron microscopy were fixed for 4.5 hours in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) at room temperature, dehydrated through an ethanol series and critical point dried. Samples were sputter-coated using gold and palladium and viewed using a Quanta 600 microscope.

In situ hybridization

In situ hybridization was carried out as described previously (Long et al., 1996; http://www.its.caltech.edu/~plantlab/protocols/insitu.htm) with the following modifications. Tissue samples were fixed in FAA for 2.5 hours at room temperature. RNase treatment of slides was left out. Substrate solution for alkaline phosphatase color reaction was prepared using 2% of a NBT/BCIP stock solution (Roche Diagnostic, Germany) in 100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$.

The *JAG* probe was transcribed using T3 RNA polymerase (Promega) from pJD37 linearized with *Hin*dIII. A *HISTONE 4* (*H4*) cDNA clone was obtained by RT-PCR amplification of *ATHH4GA* (GenBank accession number M17132) from inflorescence tissue using oJD133 and oJD134 and cloned into pBluescript-SK$^+$ (pH4-T7). *H4* probe was transcribed using T7 RNA polymerase (Promega) from pH4-T7 linearized with *Hin*dIII. *FIL* probe was transcribed using T7 RNA polymerase from pY1-Y (provided by J. Bowman) linearized with *Eco*RI.

RT-PCR

RNA isolation and reverse transcription were performed as described previously (Kardailsky et al., 1999). PCR amplification was performed on 2 μl of reverse transcription reaction. A fragment of the *JAG* gene that spans the entire coding sequence was amplified using N-0680 and N-0681. A *JGL* fragment was amplified using oJD119 and oJD120. A *TUBULIN* fragment was amplified as a control using N-1136 and N-1137. The number of PCR cycles performed is indicated in Fig. 5. Annealing temperature was 58°C for all reactions.

Results

Defects caused by loss of *JAGGED* function

We identified two T-DNA insertion alleles in the At1g68460 gene, which encodes a zinc-finger protein similar to *SUPERMAN* (*SUP*) (see below). Because of the defects in leaf and floral organ development common to both mutants, we named this gene *JAGGED* (*JAG*). *jag-1* has an insertion in intron 2, and *jag-4* in intron 4. Both alleles result in the loss of full-length *JAG* transcript as detected by RT-PCR (Fig. 5B). We focused our subsequent analysis on *jag-1*.

Inactivation of *JAG* affects the shape of most lateral organs including leaves and floral organs (Fig. 1). Rosette leaves in the

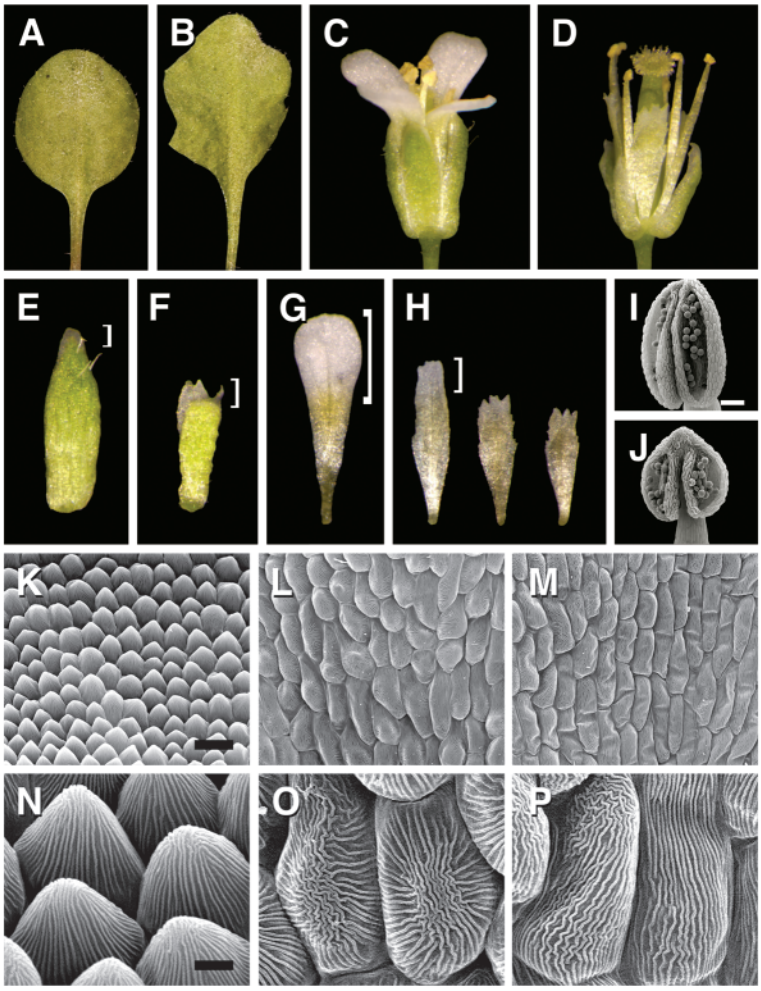

Fig. 1. Phenotypes of *jag-1* mutants. (A) Wild-type rosette leaf. (B) *jag-1* rosette leaf. (C) Wild-type flower. (D) *jag-1* flower. The sepals are shorter and narrower than those of wild type and the petals are short and narrow and do not reflect as much light as the white wild-type petals. (E) Close-up of wild-type sepal indicates small white region at the tip. (F) *jag-1* sepal. Note jagged white tissue at tip (marked by a bracket). (G) Close-up of wild-type petal. (H) Three examples of *jag-1* petals. Note that the distal white region (bracket) is reduced or absent in *jag-1*. (I,J) Scanning electron microscopy (SEM) of anthers. (I) Wild-type. (J) *jag-1*. Anthers are shorter and are spade shaped. (K-P) SEMs of petal tips. (K,N) Adaxial side of wild-type petal epidermis. The cells are uniformly conical with cuticular ridges. (L,O) *jag-1* petal cells are not as uniform and have elongated cells interspersed, which have irregular cuticular ridges. These cells resemble epidermal cells near the base of wild-type petals, shown in M,P. Scale bars: 50 μm (I,J), 20 μm (K-M) and 4 μm (N-P).

parental Ws strains are paddle shaped and have a smooth edge (Fig. 1A). *jag-1* leaves, however, develop with a serrated edge (Fig. 1B). To quantify these shape changes, the area and perimeter of rosette leaf blades were measured. While leaf blade area is not significantly different, *jag-1* leaves have a larger perimeter/area ratio (P=0.0016), reflecting the irregular blade margins of these leaves (see Table S2, http://dev.biologists.org/supplemental). All four whorls of floral organs are also affected in *jag-1*, with the defects being most apparent in sepals, petals and stamens (Fig. 1C,D). These defects are more severe in flowers produced late in development.

jag-1 sepals and petals are narrower and shorter than the wild-type organs (Fig. 1C-H). Mutant sepals develop white jagged tissue at the tip (Fig. 1E,F). Petals can be jagged at the tip, with the distal region being absent or reduced in *jag-1* (Fig. 1G,H). This region forms the part of the petal that appears white and contains cone shaped cells (Bowman, 1993). Scanning electron microscopy confirmed that the distal region of petals is absent or reduced and that only a few cells resemble wild-type cone cells (Fig. 1K,L,N,O). Often, cells at the tip of *jag-1* petals display a cuticular wax pattern and overall shape intermediate between cone cells and those at the base of wild-type petals (Fig. 1K-P).

Wild-type stamens consist of two components: the anther and the filament (Bowman, 1993). *jag-1* filaments appear normal (Fig. 1C,D), but anthers are reduced with an altered shape and typically produce less pollen than wild-type ones (Fig. 1I,J). Late flowers are often male-sterile. Carpel development is relatively normal, but under some growth conditions, surface irregularities develop along the valve walls (data not shown). Thus, *JAG* is required for the proper formation of all lateral organs, with a particularly important role in the development of the distal regions of sepals, petals and stamens.

Premature differentiation of petals in recessive *jag* mutants

Because inactivation of *JAG* has such severe effects on petal shape, we further explored the role *JAG* plays in petal development. To test whether the loss of distal petal tissue could reflect reduced cell division, we analyzed the expression of the *HISTONE 4* (*H4*) gene, which has previously been used to assay cell-cycle activity in developing tissues (Gaudin et al., 2000; Krizek, 1999; Nath et al., 2003). *H4* expression correlates with the expression of other cell-cycle markers such as *Cyclin D3b* in *Antirrhinum* (Fobert et al., 1994). As in *Antirrhinum*, at any given time only a fraction of cells show strong *H4* expression (Fig. 2B,C). In wild-type petals, the number of cells expressing *H4* increases during floral stages 7 and 8 (Fig. 2A,B) (Smyth et al., 1990). The number of *H4*-positive cells plateaus during stages 9, 10 and 11, and it begins to fall at stage 12. This peak in the number of *H4*-positive cells correlates with the rapid growth of petals during stages 9-11 (Smyth et al., 1990). *H4*-expressing cells are also much more frequent in the distal region of petals, especially during stages 8-10.

In *jag-1* petals, the number of cells expressing *H4* is normal during stages 7 and 8 (Fig. 2A-C). After stage 8, however, the number of *H4*-positive cells soon declines, and continues to decrease through stage 12. Also, the distal regions of *jag-1* petals have fewer *H4*-expressing cells than in wild type. These data, along with the localized defects of *jag-1* petals, suggest that *JAG* promotes the development of the distal region of petals by maintaining tissues in an actively dividing state.

Activation of ectopic growth in the dominant *jag-5D* mutant

We also characterized a *JAG* gain-of-function allele, *jag-5D*,

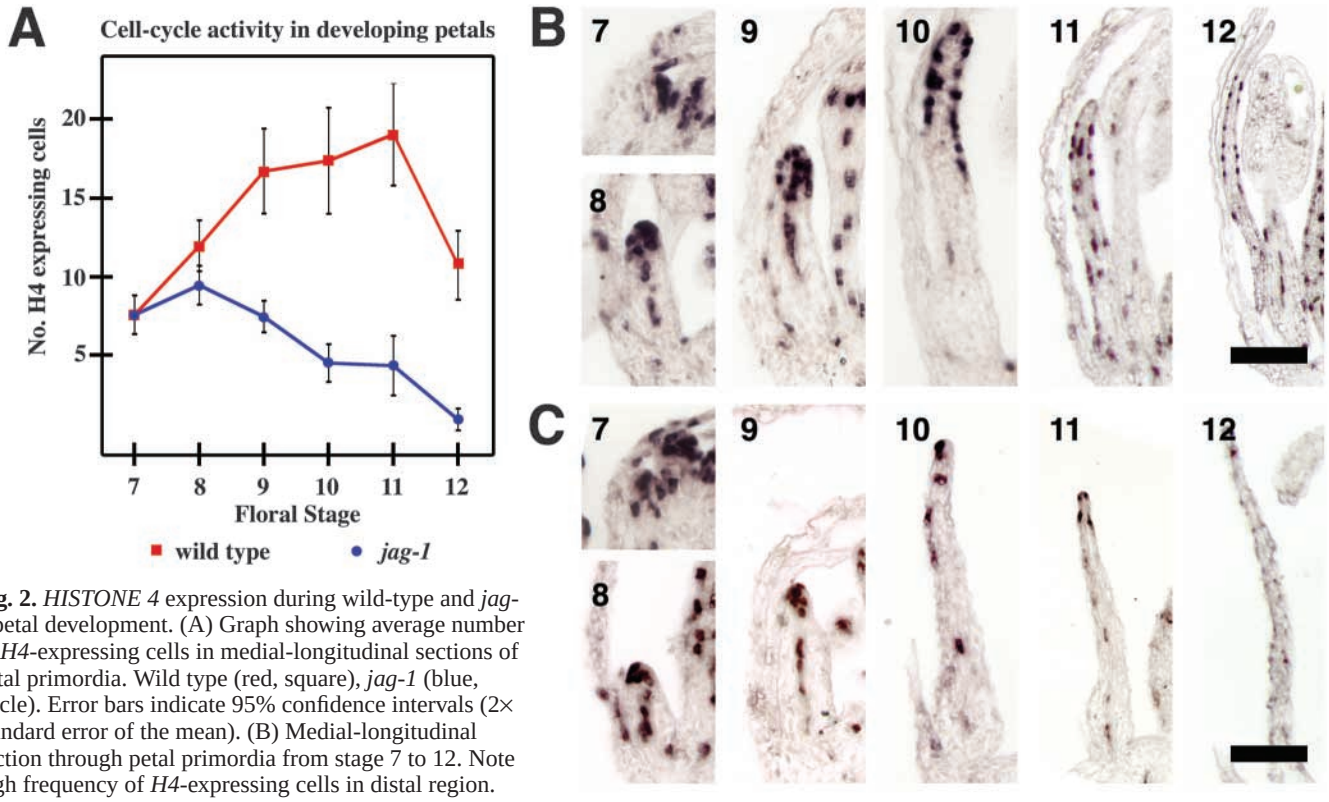

Fig. 2. *HISTONE 4* expression during wild-type and *jag-1* petal development. (A) Graph showing average number of *H4*-expressing cells in medial-longitudinal sections of petal primordia. Wild type (red, square), *jag-1* (blue, circle). Error bars indicate 95% confidence intervals (2× standard error of the mean). (B) Medial-longitudinal section through petal primordia from stage 7 to 12. Note high frequency of *H4*-expressing cells in distal region. (C) *H4* expression in petals of *jag-1* mutants. Total number of petals examined for each stage range from 8 to 17 for wild type and 8 to 26 for *jag-1*. Scale bars: 50 μm (B,C stages 7-10) and 80 μm (B,C stages 11, 12).

which was obtained using enhancer activation-tagging, in which a T-DNA with a multimerized, constitutively active enhancer is randomly inserted in the genome (Walden et al., 1994; Weigel et al., 2000). *jag-5D* mutants have a striking phenotype, with bract-like organs forming ectopically on the inflorescence, subtending most flowers (Fig. 3A-C). These leaf-like organs have stipules, lack a petiole, are pointed at their tip, and exhibit delayed senescence, confirming their identity as bracts (Fig. 3D,E, and data not shown). Using scanning electron microscopy, we found that the bracts emerge from the inflorescence meristem, with floral meristems subsequently developing in their axils (Fig. 3K,L, Fig. 6I). Thus, the bract in *jag-5D* is a product of the shoot and not the floral meristem. The degree of bract development varies from flower to flower, with alternating waves of nodes with and without bracts (Fig. 3B). The size and shape of bracts also vary, such that the bracts can be large and laminar or small and filamentous (Fig. 3D). Bracts tend to be slightly jagged (Fig. 3D), and carpelloidy is sometimes seen in late developing bracts, which are tipped with stigmatic papillae (data not shown). The *jag-5D* mutant is different from other mutants with ectopic bracts like *leafy* (*lfy*), *unusual floral organs* (*ufo*) or *filamentous flower* (*fil*) in that *jag-5D* flowers are fairly normal, although organ number is occasionally increased in the first three whorls of flowers subtended by bracts (Fig. 3A-C, and data not shown) (Levin and Meyerowitz, 1995; Sawa et al., 1999; Schultz and Haughn, 1991; Weigel et al., 1992; Wilkinson and Haughn, 1995).

The selfed progeny of the original *jag-5D* mutant fall into three phenotypic classes with a 1:2:1 ratio of plants that appear wild type, plants that develop bracts, and plants with additional

Table 1. Shoot architecture of *jag-5D* mutants

Genotype	*n*	Rosette leaves	Coflorescences (Nodes 1-20)	Leafy shoots (%)
Col-0	23	9.7±0.2	2.7±0.1	0.0±0.0
jag-5D/JAG	47	9.3±0.2	3.0±0.1	0.0±0.0
jag-5D/jag-5D	21	9.7±0.2	4.5±0.2	17.6±1.5

Values are mean±s.e.m.

replacement of some flowers by leafy shoots (Fig. 3A-C,F; Table 1). Genotyping and progeny testing determined that the latter are homozygous for *jag-5D*. Bracts are more prominent and develop more often in these plants (Fig. 3B,C). In addition, the number of coflorescences (side shoots with flowers) that form on the main inflorescence is increased, and coflorescences are occasionally replaced by shoots containing only bracts (Fig. 3F, Table 1). These leafy shoots most frequently develop on the basal nodes of the inflorescence; occasionally, they interrupt a series of flowers. Replacement of flowers with coflorescences is less frequent in *jag-5D* heterozygotes (Fig. 3G). These phenotypes suggest that *JAG* can suppress floral meristem identity, which leads to a copy number-dependent conversion of flowers into coflorescences or leafy shoots.

The *jag-5D* mutation also affects the growth of rosette leaves (Fig. 3H,I). In wild type, the petiole is most defined in the first rosette leaves and gradually becomes reduced leading up to the cauline leaves. In both homozygous and heterozygous *jag-5D* mutants, the petiole is either absent or develops blade tissue along its edge, creating a teardrop shape. Homozygous mutants have slightly more blade tissue on the petiole than

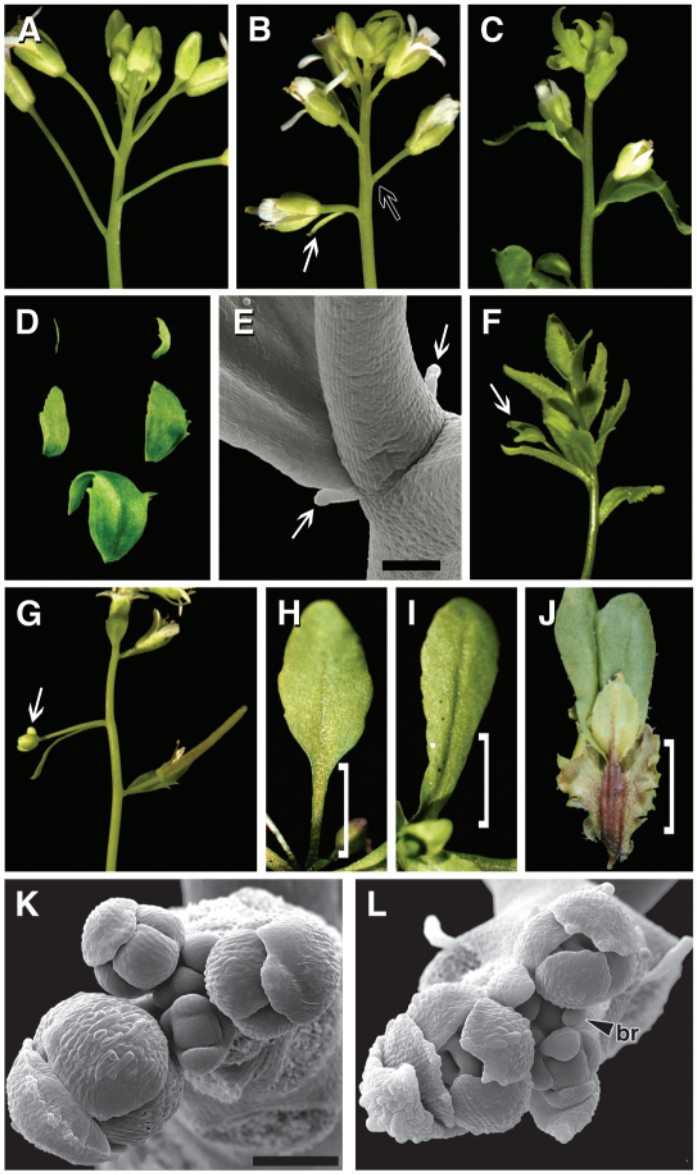

Fig. 3. Phenotypes of *jag-5D* mutants. (A-C) Inflorescences of wild-type (A), *jag-5D* heterozygous (B), and *jag-5D* homozygous plants (C). White arrow points to flower subtended by a bract, black arrow points to a bractless flower. (D) Examples of bract development on the inflorescence of *jag-5D* heterozygous mutants including filamentous, (top left), and large laminar bracts (bottom center). Note jagged edges and pointed tips of bracts, and lack of defined petioles. (E) SEM of stipules at the base of a bract of a *jag-5D* homozygous mutant (arrows). (F) Example of suppression of floral identity in a *jag-5D* homozygote. Arrow points to a leafy shoot. (G) Example of ectopic co-florescence in *jag-5D* heterozygote (arrow). (H) Wild-type rosette leaf with typical paddle shape. Bracket marks petiole. (I) In *jag-5D* mutants, blade tissue develops ectopically on petiole. (J) Ectopic blade tissue on the stem of shoots that develop in the axils of rosette leaves (bracket marks ectopic blade). (K) SEM of a wild-type inflorescence apex. (L) SEM of a *jag-5D* heterozygote. Arrowhead points to a bract primordium (br). Scale bar: 100 μm (E,K,L).

heterozygotes, although it is difficult to distinguish them on this basis (data not shown).

We also found ectopic blade tissue on the stems of coflorescences that arise in the axils of rosette leaves (Fig. 3J). This blade tissue appears to be continuous with that of the first leaves of the coflorescence. The development of blade tissue on rosette leaves and stems shows that *jag-5D* can activate ectopic growth of tissue and suggests that this activity of *JAG* may be the cause of ectopic bract development, as well.

Structure of the *JAG* gene

The *JAG* gene was initially identified starting with the *jag-5D* mutant, which contains a single activation-tagging T-DNA inserted downstream of the annotated gene, At1g68480. *JAG* encodes a putative single zinc-finger of the C_2H_2 type, with high sequence similarity (35% identity at amino acid level) to its closest characterized *Arabidopsis* homolog, *SUP* (Sakai et al., 1995). To confirm the identity of the *JAG* gene, a genomic fragment including the At1g68480 coding sequence and *35S* enhancer sequences from the activation-tagging T-DNA was transformed into wild-type plants (Fig. 4A). 53 of 150 primary transformants showed the *jag-5D* phenotype to various degrees, including plants that had a stronger phenotype than the original *jag-5D* mutant (Fig. 4B and data not shown). We were also able to complement *jag-1* mutants with a similar genomic fragment that lacked the 35S enhancer sequences (data not shown).

JAG has a close homolog in *Arabidopsis*, At1g13400, which we named *JAGGED-LIKE* (*JGL*) (53% identity). A *JAG*-related sequence was also found in rice, represented by an EST, which we named Os*JAG* (GenBank accession numbers C26936, C50482). *JAG* and *JGL* form a well-supported monophyletic group, with Os*JAG* as sister group (data not shown). Alignment of JAG, JGL, OsJAG and SUP proteins indicates very similar sequences in the C_2H_2 zinc-finger, including the plant-specific QALGH motif (Fig. 4D) (Takatsuji, 1998). Some of the residues that provide DNA binding sequence specificity in the C_2H_2 zinc-finger region differ between SUP and JAG, JGL and OsJAG, which all share identical residues at these positions, suggesting that the JAG-like proteins bind to other sequences than SUP (Takatsuji, 1996) (Fig. 4D). A proline-rich region C-terminal to the zinc-finger may function as a protein-protein interaction domain (Falquet et al., 2002; Kay et al., 2000). While the EAR motif [ERF-associated amphiphilic repression motif (Hiratsu et al., 2002)] of SUP is only weakly conserved in JGL and OsJAG, there is a similar motif in JAG (Fig. 4D). At the N terminus, the JAG-like proteins contain a motif that distinguishes them from SUP. In addition, JAG contains a putative monopartite nuclear localization signal (NLS) near the N terminus (Fig. 4D) (Kalderon et al., 1984). We confirmed the predicted subcellular localization of JAG with a GFP fusion, which localizes to the nucleus (Fig. 4C).

Expression pattern of *JAG*

By RT-PCR, *JAG* RNA is detected during vegetative and reproductive development in the shoot apex and open flowers, but not in roots, leaves or inflorescence stems (Fig. 5A). As expected, *JAG* mRNA levels are increased in *jag-5D* mutants (Fig. 5B). *JGL* mRNA is found in the same tissues where *JAG* RNA accumulates (Fig. 5A). The overlap in RNA accumulation along with the high degree of sequence similarity

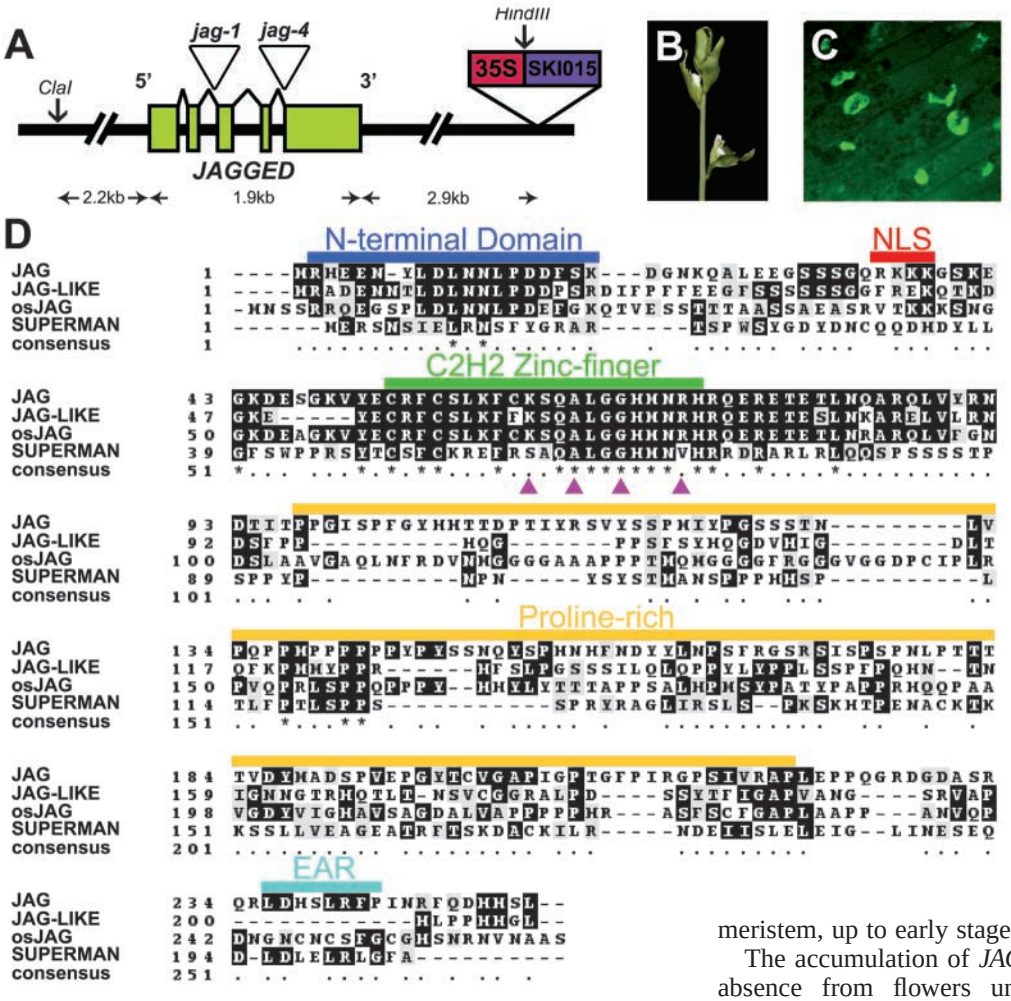

Fig. 4. Molecular characterization of *JAG*. (A) Genomic structure. Small vertical arrows indicate borders of restriction fragment used for recapitulation (see B). Triangles mark T-DNA insertions in *jag-1* and *jag-4*. (B) Inflorescence of an intermediate line that recapitulates the *jag-5D* phenotype. (C) Confocal image of epidermal cells from the pedicel of a floral primordium transformed with a construct in which a *JAG:GFP* fusion protein is expressed under control of the *AP1* promoter. (D) Alignment of predicted amino-acid sequences of JAG, JGL, OsJAG and SUP. Periods indicate residues that are identical or similar in at least two sequences, and small asterisks indicate residues that are identical in all sequences. Beneath the zinc-finger domain, pink arrows indicate residues that determine DNA-binding specificity.

between *JAG* and *JGL* suggests that the two genes are partially redundant.

A more detailed picture of *JAG* gene activity was obtained by in situ localization of *JAG* mRNA. During the vegetative phase, *JAG* mRNA is detected in emerging leaf primordia, but is absent from the shoot apical meristem (Fig. 6A). *JAG* mRNA is localized in the distal region of leaves (Fig. 6A). Serial sections through leaf primordia revealed that *JAG* is predominantly expressed in the blade regions (Fig. 6B,C). No *JAG* expression was detected in the petioles (Fig. 6B). During the reproductive phase, *JAG* mRNA is excluded from the inflorescence and floral meristem, up to early stage 2 of floral development (Fig. 6E).

The accumulation of *JAG* mRNA in leaf primordia, but its absence from flowers until mid-stage 2, contrasts with expression of *FIL*, another lateral organ marker that is detected not only in emerging leaves, but also in cells on the abaxial side of stage-2 floral primordia (Fig. 4D) (Siegfried et al., 1999). These cells, from which *JAG* mRNA is notably absent, constitute the cryptic bract (Long and Barton, 2000).

Like *FIL*, *JAG* mRNA is expressed in initiating sepal, petal, stamen and carpel primordia (Fig. 6E,F). Accumulation of *JAG* mRNA in sepals and stamens declines soon after stage 6, while expression in the distal region of the petal is maintained through stage 11 (Fig. 6H). This expression domain overlaps with the region of the petal that has the highest proportion of *H4*-positive cells (Fig. 2B). In the carpel, *JAG* mRNA initially accumulates throughout the valves during stages 7 and 8 (Fig. 6G). By stage 9, *JAG* mRNA accumulates primarily near the valve margins and gradually disappears by stage 12 (Fig. 6G). The *JAG* signal seems somewhat patchy in most tissues, with some cells apparently accumulating more *JAG* transcript than others.

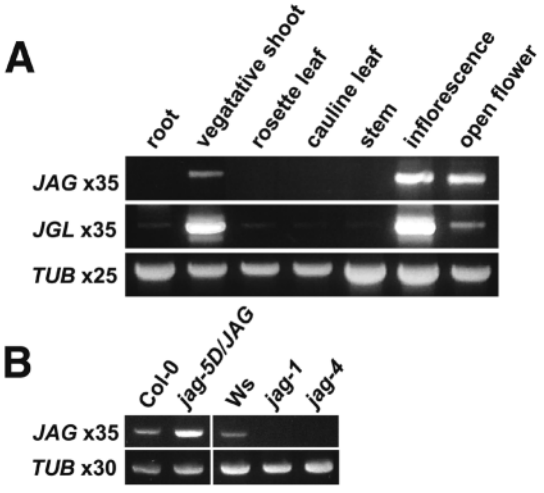

Fig. 5. RT-PCR analysis of *JAG* and *JGL* expression in different tissues. (A) Expression in wild type. Numbers refer to PCR cycles. RNA was extracted from roots of seedlings grown vertically on MS agar plates; vegetative shoots of 2-week-old plants; mature rosette leaves; mature cauline leaves; inflorescence stems; 4-week-old inflorescence shoots including flowers up to stage 12; and stage 13 open flowers. (B) *JAG* expression in inflorescences of *jag* mutants. Amplification of *TUBULIN* was used as a control.

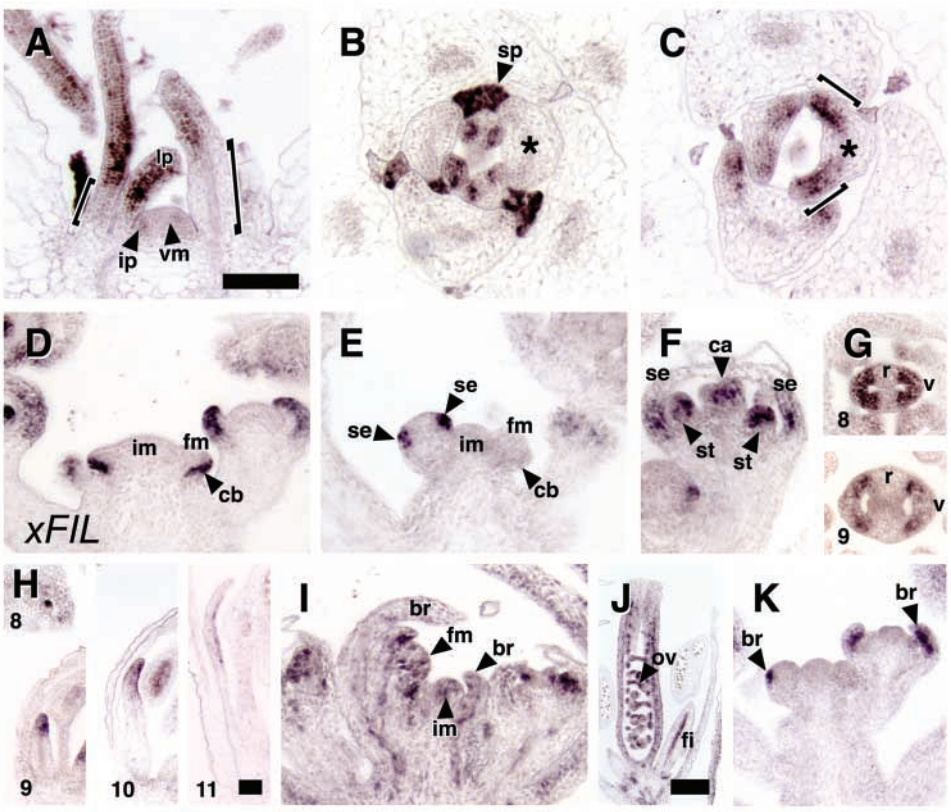

Fig. 6. Expression of *JAG* and *FIL*, examined by in situ hybridization. (A-C, E-H) *JAG* expression in wild-type Columbia. (A) Vegetative shoot apex from a 2-week-old plant. *JAG* is expressed in incipient leaf primordia (ip) and older leaf primordia (lp), but absent from the shoot apical meristem (vm) and petiole regions (brackets). (B,C) Serial transverse sections of a vegetative shoot separated by 16 μm. *JAG* is expressed in the leaf blade and is strongest in the marginal regions (brackets) and excluded from the petiole. Asterisk marks same leaf in both sections. Strong *JAG* expression is also seen in stipules (sp). (D) *FIL* expression in wild-type Columbia inflorescence. Arrow indicates the cryptic bract (cb). Inflorescence meristem (im), floral meristem (fm). (E) *JAG* is expressed very early in the presumptive sepal (se) primordia of late-stage 2 flowers. *JAG* expression is not detected in floral or inflorescence meristems or the cryptic bract. (F) Stage 6 floral primordia show strong expression in initiating stamens (st), carpels (ca) and fading expression in sepals. (G) Transverse section of gynoecium. Floral stage indicated in lower left corner. During stage 8, *JAG* is expressed in the valves (v) and excluded from the replum (r). At stage 9, *JAG* expression diminishes in the valves but is maintained near the valve margins. (H) Expression of *JAG* during petal development from stage 8 to 11. *JAG* accumulates in the distal region of petals and disappears by stage 12. (I,J) *JAG* expression in *jag-5D* heterozygotes. (I) Note weak signal throughout the inflorescence, with higher levels of expression in the initiating bract (br) and inflorescence meristem. (J) Prolonged and ectopic expression in stamen filament (fi), and gynoecium including ovules (ov) in a stage 12 flower. (K) *JAG* is strongly expressed in the ectopic bracts of *ap1* mutants. Scale bars: 100 μm (A-G, I, K), 50 μm (H), and 200 μm (J).

That *JAG* is expressed in leaf primordia, but excluded from cryptic bracts suggests that ectopic expression of *JAG* in the cryptic bract of *jag-5D* mutants leads to ectopic bract development. Consistent with this hypothesis, *JAG* RNA accumulates in the initiating bracts of *jag-5D* heterozygotes (Fig. 6I), where it is maintained long after emergence of the bracts. *JAG* RNA is also detected in the mutant in other tissues in which it is normally not expressed, such as the inflorescence meristem and ovules (Fig. 6I,J). Finally, *JAG* mRNA also accumulates in the ectopic bracts that replace first-whorl sepals in *apetala1* (*ap1*) mutants (Fig. 6K) (Bowman et al., 1993; Irish and Sussex, 1990).

Requirement of *JAG* for bract development in *ap1-15* mutants

The appearance of bracts in *jag-5D* mutants is of special interest, since *Arabidopsis* normally does not produce such organs, although, as mentioned above, ectopic bracts are produced in flowers of strong *ap1* mutants. To determine whether *JAG* is not only sufficient, but also necessary for bract formation, we introduced the *jag-1* loss-of-function allele into the *ap1-15* background. Most *jag-1 ap1-15* flowers do not develop any organs in the first whorl (Fig. 7A-D, Table 2). Bracts are sometimes replaced by rudimentary organs that are similar to those found in late-arising *ap1-15* flowers (data not shown). Scanning electron microscopy showed that early bract development is similar in *ap1-15* and *jag-1 ap1-15* flowers (Fig. 7E,F). In *ap1-15* flowers, medial bracts continue to grow, while lateral bract primordia typically abort. In *jag-1 ap1-15* flowers, both medial and lateral bracts abort soon after initiation. Importantly, other aspects of the *ap1-15* mutant phenotype are unaffected by *jag-1* (Fig. 7C,D, Table 2). Thus, *JAG* is required for the outgrowth of bracts in *ap1* mutants, but dispensable for their initiation. This is consistent with the finding that cryptic bracts in wild-type plants also initiate independently of *JAG*.

Effects of flower-specific ectopic *JAG* expression

In order to determine the effects of over-expressing *JAG* specifically in developing flowers, we used the *AP1* promoter, which is active in flower primordia from stage 1 on (Hempel et al., 1997). *AP1::JAG* plants produce a range of phenotypes. In the weakest lines, organs in the first three whorls are fused (Fig. 8D). Typically, sepals are connately fused at their base. Both petals and stamens fuse to form abnormal organs that consist predominantly of petal tissue. In intermediate lines, the severity of sepal fusion increases (Fig. 8A,E). In the most extreme cases, a tube of sepal tissue sheathes the inner whorls of the flower (Fig. 8E). The development of the tissue interior to the sepals is often reduced, although carpels can occasionally be seen developing in the center of the flower (Fig. 8E). Another peculiarity of these flowers is that sepals are not clearly distinct from the pedicel, and that sepal tissue

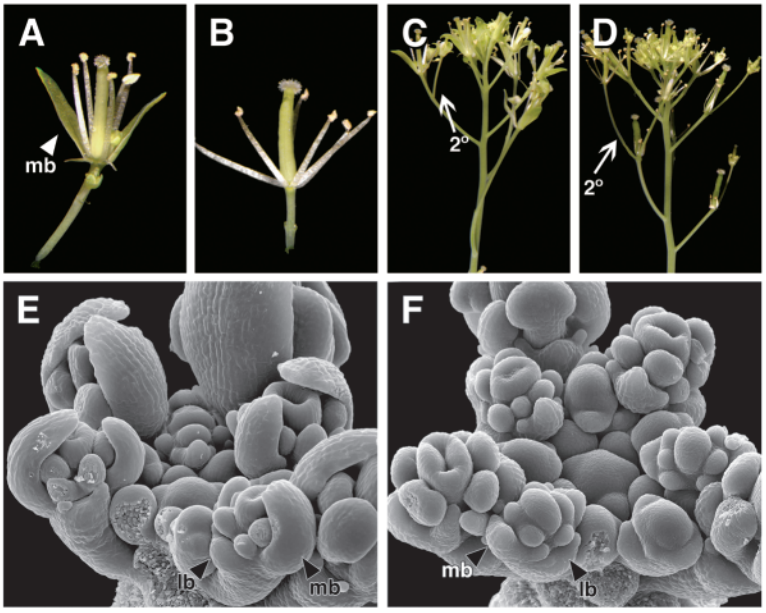

Fig. 7. Phenotypes of *jag-1 ap1-15* double mutants. (A) *ap1-15* flower, with a medial bract (mb) indicated. (B) *jag-1 ap1-15* flower, lacking all bracts. (C) *ap1-15* inflorescence, with secondary flowers (2°) indicated. (D) *jag-1 ap1-15* inflorescence; note that secondary flowers are unaffected. (E) SEM of *ap1-15* inflorescence apex. In *ap1-15* mutants, lateral bracts (lb) fail to grow, while medial bracts continue to grow. (F) SEM of *jag-1 ap1-15* inflorescence apex. Both lateral and medial bracts fail to grow out.

extends down the length of the pedicel to the inflorescence stem (Fig. 8A,E-G).

In the strongest *AP1::JAG* lines, flowers are replaced by snake-like stumps that do not develop any organs (Fig. 8B,C). First-whorl organs seem to initiate, but they fail to grow. Organs of the inner whorl either do not initiate or, if there is any growth, grow amorphously and do not resemble any particular organ type (Fig. 8H). We confirmed by RT-PCR that the phenotypic severity of our *AP1::JAG* lines correlated with increased levels of *JAG* transcript (see Fig. S2, http://dev.biologists.org.supplemental).

To determine the identity of the structures that develop in the place of flowers in strong *AP1::JAG* lines, we examined the expression of *FIL*, which, in stage 3 flower primordia, marks discrete populations of cells that develop into sepals (Siegfried et al., 1999). In strong *AP1::JAG* lines, *FIL* expression extends down the length of the snake-like outgrowths, suggesting that these structures have at least partial lateral organ identity (Fig. 8I,J).

In aggregate, the phenotypes caused by the *AP1::JAG* transgene suggest that *JAG* can override the arrest or delay in the growth of several tissues. In weak and intermediate lines, growth is activated between organs leading to fusion of sepals, petals and stamens. In intermediate lines, precocious growth of sepals leads to an elongated sepal tube. In the strongest lines, growth may be initiated before floral organs are properly established leading to the outgrowth of stumps that lack any discrete organ development.

Table 2. Flower architecture of *ap1-15* and *jag-1 ap1-15* double mutants

			First-whorl	Second-whorl organs		
Genotype	*n*	2° flowers	bracts	Stamens	Petals	Other
ap1-15	97	2.2±0.1	1.5±0.1	3.7±0.1	0.0±0.0	0.8±0.1
jag-1 ap1-15	77	2.4±0.1	0.1±0.0	4.1±0.1	0.0±0.0	0.6±0.1

Values are mean±s.e.m. Flowers 1-10 were counted.

Discussion

We have described the function and expression of the *JAG* gene, an important regulator of growth in lateral organs of *Arabidopsis*. *JAG* can activate growth of many different tissue types, including bracts, which are suppressed in wild-type plants. The absence of *JAG* expression from the cryptic bracts of wild-type plants contrasts with expression in most other lateral organs, suggesting a possible cause for bractless flowers in *Arabidopsis* and its relatives.

The role of *JAG* in lateral organ development

The plant shoot apex is a dynamic structure in which a meristem maintains a population of undifferentiated stem cells that later undergoes a process of differentiation to form lateral organs. This process involves several steps, beginning with the loss of meristem identity and the acquisition of lateral organ identity, followed by organ outgrowth and morphogenesis, then cell-cycle arrest and finally histogenesis. Progress through differentiation, however, must be regulated so that organs can be properly shaped. If tissues exit the cell cycle prematurely, morphogenesis is incomplete, whereas if cell-cycle arrest does not occur, lateral organs would continue to grow ad infinitum.

In *jag* loss-of-function mutants, lateral organs do not develop completely, with the strongest defects in the distal regions of organs. In petals, we have shown that this is probably due to a reduction in cell-cycle activity. In eudicots, lateral organs typically differentiate in a basipetal fashion with cells at the tip exiting the cell cycle before those at the base (Donnelly et al., 1999; Nath et al., 2003; Poethig and Sussex, 1985). We propose that *JAG* slows the cessation of cell division in the distal region of organs until proper morphogenesis has occurred, which is particularly apparent in floral organs, since these form different structures along the proximal-distal axis. *JAG* is also expressed in the blade region of leaves, and *jag* loss-of-function mutants develop serrated leaves. While we did not detect a change in total leaf area, the serrated leaves may be caused by a reduction in growth in the inter-hydathode regions of the blade. In this regard it is interesting to note that plants that overexpress *ICK1* or *KRP2*, negative regulators of the cell cycle, also have jagged leaf blades (De Veylder et al., 2001; Wang et al., 2000). This interpretation is also consistent with *jag-5D* gain-of-function phenotypes in which rosette leaves develop ectopic blade tissue along the petiole. It is an intriguing concept that the shape of organs and the architecture of shoots in plants may be, in part, the result of controlled differentiation of tissues. Thus, the plant may be sculpted by the opposing inputs from genes like *CIN* that promote cell-cycle arrest, and genes like *JAG* that suppress cell-cycle arrest.

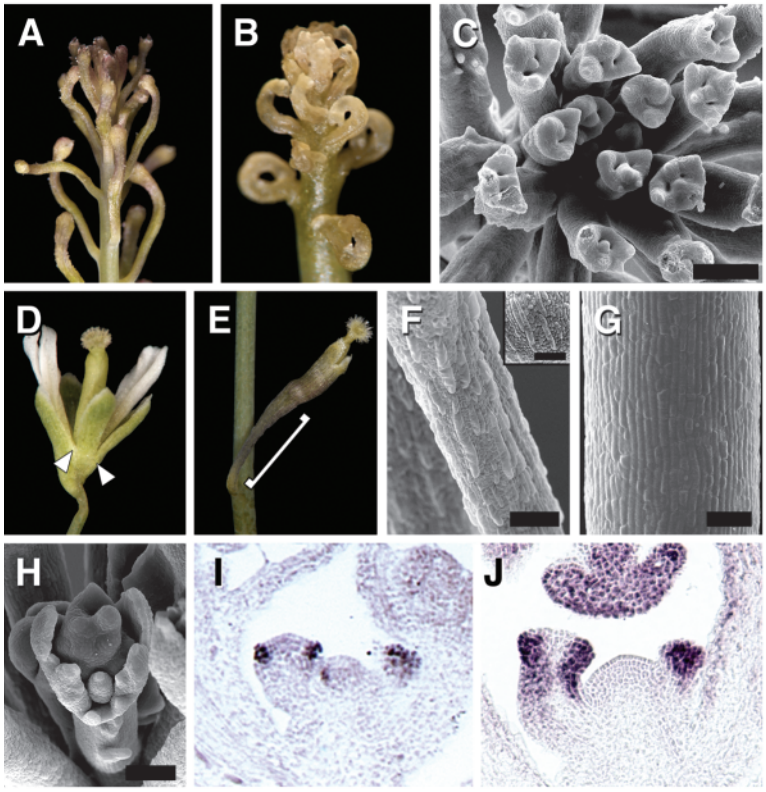

Fig. 8. Phenotypic effects of *JAG* expression under the control of the *AP1* promoter. (A,E,F) Intermediate *AP1::JAG* lines usually form flowers with extended sepal-tubes sheathing the inner whorls. The junction between sepals and the pedicel is abnormal with sepaloid tissue running down the length of the pedicel (indicated by bracket in E). (F) SEM shows that the epidermis of the stalk of *AP1::JAG* flowers resembles that of wild-type sepals, shown in the inset. It differs from the wild-type pedicel epidermis shown in (G). (B,C,H) Strong *AP1::JAG* lines develop snake-like outgrowths in place of flowers. Only first-whorl organs initiate. (H) Amorphous tissue can sometimes be seen developing in the center of flower-like structures. (D) Weak *AP1::JAG* lines typically have sepals that are fused at the base (arrows). In place of petals and stamens, composite organs with petal and stamen identity form. (I) Expression of *FIL* in a stage 3 floral primordium of wild type. (J) In a strong *AP1::JAG* line, the *FIL* domain is expanded into the pedicel. Scale bars: 200 μm (C), 100μm (F,H) and 50 μm (G).

One of the most dramatic consequences of *JAG* over-expression in *AP1::JAG* plants is the inhibition of floral organ boundaries and the precocious growth of sepals creating tube- and snake-like structures. These phenotypes suggest that increased *JAG* levels can override factors that inhibit the growth of specific organ regions. That *JAG* over-expression has such dramatic consequences even though *JAG* is normally expressed in young organs indicates that expression levels need to be finely tuned for proper function of *JAG*.

Bract development in *Arabidopsis*

One of the characteristic features of members of the Brassicaceae family is that most flowers lack bracts, which is in stark contrast to the presence of these organs in most eudicots. However, bracts are not completely absent in Brassicaceae as basal flowers in many species are subtended by bracts (Arber, 1931). Although bracts are normally absent in *Arabidopsis*, they can develop in certain backgrounds, such as *lfy*, *ufo* or *fil* mutants. Less pronounced activation of cryptic bracts is seen when flowers are ablated through expression of a toxin (Nilsson et al., 1998). Even in wild-type plants, molecular markers indicate the presence of a cryptic bract whose outgrowth is normally suppressed (Long and Barton, 2000). Thus, while the bract appears to be dispensable for the final architecture of the *Arabidopsis* inflorescence, the bract is still patterned.

In *Arabidopsis*, several genes, *FIL*, *AINTEGUMENTA*, *ASYMMETRIC LEAVES1* and *STYLISH1*, are expressed in incipient lateral organs (Byrne et al., 2000; Kuusk et al., 2002; Siegfried et al., 1999). At least some of these genes are required for the development of lateral organs, but none is sufficient to activate bract development, even though they are expressed in the cryptic bract. Thus, their activity is either specifically suppressed in the cryptic bract, or bract outgrowth requires another factor. The latter scenario is supported by the finding that *JAG* is absent from the cryptic bract, but can activate its outgrowth when ectopically expressed in *jag-5D* mutants. That this function does not merely represent a neomorphic activity is demonstrated by our observation that *JAG* is required for outgrowth of ectopic bracts that form in *ap1-15*. It is particularly interesting that loss of *JAG* function in an *ap1-15* background primarily affects bracts, even though it is also expressed in other organs. This may indicate that *JAG* is more important for the growth of bracts, and that redundancy with a gene such as *JGL* may mask essential functions in other organs. Alternatively, *ap1* bracts may be particularly sensitive to defects in organogenesis. This could be the case if *ap1* bracts are under similar suppressive influences as bracts of the main inflorescences. In this case, loss of *JAG* would sensitize the bract to suppression of outgrowth. This interpretation is further supported by the observation that loss-of-function in another gene important for lateral organ development, *AINTEGUMENTA*, also has a negative effect on bract development in *ap1* mutants (B. Krizek, personal communication).

Since our data suggest that *JAG* is both necessary and sufficient for bract formation, and since *JAG* expression is normally excluded from the cryptic bract, one can speculate that bract-specific suppression of *JAG* expression is the cause of bractless flowers in the Brassicaceae. As there are also other examples of bract suppression in plants outside of Brassicaceae, such as in the maize inflorescence (Ritter et al., 2002), it will be interesting to determine the possible role that *JAG* plays in the evolution of this intriguing plant characteristic in other species, as well.

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Table S1. Oligonucleotides

Oligonucleotide name	Sequence 5′ to 3′
JL202	CAT TTT ATA ATA ACG CTG CGG ACA TCT AC
N-0335	CAA GAG ACA ATG ATT CG
N-0336	AGT ACA CAT CCA TGA TG
N-0680	AAG CTT CTC TCA TTT CCC ACT ATG AGG
N-0681	GGA TCC AAC TCA GAG CGA GTG ATG ATC TTG
N-1136	GGA CAA GCT GGG ATC CAG G
N-1137	CGT CTC CAC CTT CAG CAC C
N-1365	AGG ATC ACA AGT TCA TCA AGG ACC ATC TT
N-1366	ATT AAT TTG TCC GGT TCC TCT CTG TCC TC
N-1413	CTG CAG CGA GCG AGT GAT GAT CTT GAA ACC
N-1509	TT TAG CTA CGC ATG TGG TCA
N-1510	TGT GGT GGA TAA CGG ACG TA
oJD119	AAG CTT ATG AGA GCT GAT GAA AAT AAC AC
oJD119	AAG CTT ATG AGA GCT GAT GAA AAT AAC AC
oJD120	GGA TCC TTA TAG CCC ATG ATG TGG AGG
oJD120	GGA TCC TTA TAG CCC ATG ATG TGG AGG
oJD126	TTA GTT TCC ACA CGC AGA GAG AG
oJD127	TCA TGT GGC CAC CAA GAG CTT G
oJD133	ACA CGG ATC GAT ATC TCA GCC
oJD134	CAC CGA ATC CGT AAA GAG TCC
SKC12	TTG ACA GTG ACG ACA AAT CG

Table S2. Measurements of wild-type and *jag-1* rosette leaf blades

Genotype	*n*	Area (mm^2)	Perimeter (mm)	Perimeter/Area (mm^{-1})
Col-0	24	70.51±1.55	30.94±0.36	0.44±0.00
jag-1	25	69.84±2.24	32.63±0.57	0.47±0.00

Measurements were taken from the third and fourth rosette leaves.
Values are mean±s.e.m.