The *Arabidopsis* JAGGED gene encodes a zinc finger protein that promotes leaf tissue development

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**Summary**

Important goals in understanding leaf development are to identify genes involved in pattern specification, and also genes that translate this information into cell types and tissue structure. Loss-of-function mutations at the JAGGED (JAG) locus result in Arabidopsis plants with abnormally shaped lateral organs including serrated leaves, narrow floral organs, and petals that contain fewer but more elongate cells. jag mutations also suppress bract formation in leafy, apetala1 and apetala2 mutant backgrounds. The JAG gene was identified by map-based cloning to be a member of the zinc finger family of plant transcription factors and encodes a protein similar in structure to SUPERMAN with a single C2H2-type zinc finger, a proline-rich motif and a short leucine-rich repressor motif. JAG mRNA is localized to lateral organ primordia throughout the plant but is not found in the shoot apical meristem. Misexpression of JAG results in leaf fusion and the development of ectopic leaf-like outgrowth from both vegetative and floral tissues. Thus, JAG is necessary for proper lateral organ shape and is sufficient to induce the proliferation of lateral organ tissue.

Key words: JAGGED, Lateral organ growth, Leaf morphogenesis, Leaf development, *Arabidopsis thaliana*

**Introduction**

Lateral organs (leaves and floral organs) develop as outgrowths from the flanks of the shoot apical meristem (SAM). During their growth, cell division and differentiation must be coordinated along three principal axes: a proximal-distal axis, along which the proximal petiole and the distal blade are positioned; an adaxial-abaxial axis defining the position of tissues that abut the meristem versus those located farther away; and a medial-lateral axis that defines the position of the central midrib relative to the lateral blade and leaf margins. Recent studies have uncovered a large number of genes that specify adaxial/abaxial patterning including PHANTASTICA (PHAN) (Waite and Hudson, 1995; Waite et al., 1998), PHABULOSA and PHAVOLUTA (PHB and PHV) (McConnell and Barton, 1998; McConnell et al., 2001), ARGONAUTA (AGO) (Bohmert et al., 1998), PINHEAD (PNH/ZWILLE) (Moussian et al., 1998; Lynn et al., 1999; Newman et al., 2002), FILAMENTOUS FLOWER (FIL)/YABBY genes (Siegfried et al., 1999) and KANADI1/KANADI2 (KAN1/KAN2) (Eshed et al., 2001; Kerstetter et al., 2001). When these genes are mutated, lateral organs frequently develop as radially symmetrical structures, demonstrating the importance of adaxial-abaxial polarity for wild-type leaf blade formation.

In response to positional cues, genes presumably dictate the process of morphogenesis by controlling both the proliferation and the differentiation of specific cell types. Studies of leaf growth in *Arabidopsis* using genetic clonal analysis (Poethig, 1985) and observation of a *cyclin1A*:β-glucuronidase (GUS) cell cycle reporter gene (Donnelly et al., 1999) showed that in growing leaves there is a proximal-distal gradient of cell division rates, with the highest levels of cell division occurring in the proximal regions of the leaf. In contrast, most leaves differentiate basipetally with the distal blade forming first, followed by the proximal petiole (Poethig, 1985). Recent studies have identified a number of genes involved in the control of cell division and differentiation in lateral organs. The LEAFY PETIOLE (LEP) gene encodes an EREBP/AP2-type transcription factor that is sufficient to transform the proximally located leaf petiole into a distal blade structure (van der Graaff et al., 2000). Additional REPRESSOR FOR LEP (RLP) loci have been isolated as suppressors of an activation-tagged *lep* mutant (van der Graaff et al., 2003). Mutations in the BLADE-ON-PETIOLE 1 (BOP) locus result in abnormal leaves that misexpress the meristem-promoting knox genes and develop numerous leaf primordia on the adaxial surface of the leaves (Ha et al., 2003). *asymmetric leaves1* (as1) and *asymmetric leaves2* (as2) mutants also misexpress *knox* genes in leaves (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). Finally the AINTEGUMENTA (ANT) gene, encoding an AP2-like factor, is proposed to control organ size by regulating meristematic competence of cells within the lateral organ primordia (Krizek, 1999; Mizukami and Fischer, 2000).

We show that loss-of-function *jagged* (jag) mutants exhibit lateral organ defects, including narrow leaves and floral organs with serrated margins. The JAG locus encodes a nuclear-localized transcription factor containing a single C2H2-type zinc finger that is expressed exclusively in developing lateral organ primordia. When expressed ectopically, JAG activity is sufficient to cause cell proliferation and differentiation of leaf cell types.
Materials and methods

Plant growth conditions

Plants were grown in a (4:3:2) mixture of potting soil:vermiculite:perlite in a 22°C growth chamber with continuous cool-white fluorescent lights.

Genetic analysis of mutants

The jag-2 and jag-3 alleles (isolation lines, shredde1 and shredde2) arose in an EMS screen. Landsberg erecta (Ler) seed were mutagenized by incubation in a solution containing 0.3% (v/v) EMS (Sigma) and 0.1% (v/v) Tween 20 (Sigma) for 12 hours at room temperature. Both alleles were out crossed to wild type four times before further genetic analysis. For the construction of double mutant strains, the progeny of single mutant phenotype individuals were collected in the F2 generation and the double mutants were observed to segregate in a 1:3 ratio in the F3 generation. Double mutant genotypes were confirmed by polymerase chain reaction (PCR) genotyping.

Scanning electron and confocal microscopy

For SEM the inflorescence tissue was fixed and processed using a standard protocol (Bowman et al., 1989). Stages of flower development (Smyth et al., 1990) were determined. Stage 13 flowers (anthesis stage) were selected for SEM. Petals were carefully removed and mounted flat either on their adaxial or abaxial surface. For confocal analysis, 4-day post-germination seedlings were immersed in 1 mg/ml Hoechst 33342 for 1 hour. After removal of one cotyledon, seedlings were mounted in 50% glycerol under a coverslip. Tissue was imaged using a Zeiss LSM510 NLO confocal microscopy equipped with a Chameleon Ti-Sapphire IR pulsed laser (Coherent) tuned to 730 nm. JAG-VENUS was detected using the 514 nm Ar laser line with a 435-485 band pass filter for the emitted light. The two signals were collected together using multi-tracking and the images were analyzed with Zeiss LSM510 proprietary software and ImageJ (http://rsb.info.nih.gov/ij/).

Histology and plastic sections

Clearing of tissues for observation of vasculature by light microscopy was performed as described previously (Aida et al., 1997).

For plastic sections tissue was vacuum infiltrated with a fixation solution containing 4% paraformaldehyde, 1% glutaraldehyde and 1× phosphate-buffered saline and fixed for 12 hours at 4°C. Tissues were dehydrated stepwise in ethanol solution series. Tissues were infiltrated stepwise in Unicryl™ acrylic resin (SPI Supplies) diluted with ethanol. Samples were polymerized for 20-24 hours in a 60°C oven. Sections 0.2-2.0 μm were cut with a glass blade and a Leica RM2165 microtome. Samples were stained with 1% Toluidine Blue for 1 minute and mounted in Cytoseal-XYL (Richard-Allan Scientific).

Positional Cloning of JAG

To create a mapping population, the jag-3 allele in Ler ecotype was crossed to wild-type Columbia (Col) ecotype plants. Recombinant jag-3 mutant plants were identified in the F2 progeny. PCR-based markers were developed for fine scale mapping using Ler and Col single nucleotide and insertion/deletion polymorphism sequence data located at http://www.arabidopsis.org/Cereon/index.html.

For genomic complementation of the mutant with genomic constructs P5112-1 and P5112-4, overlapping 10-15 kb genomic fragments spanning the BAC T26H14 were subcloned into the pPZP222 transformation vector (Hajdukiewicz et al., 1994) for transformation of jag-3 plants by Agrobacterium-mediated transformation by the floral dip method (Clough and Bent, 1998). The 1.2 kb JAG cDNA was PCR-amplified from reverse transcribed Ler inflorescence total RNA using the primers P5.9f 5'-CCCTAGCAGTCTCCCTTACCTCAG-3' and P5.2r 5'-GGGCTTTGACATA-TCTTAGATCTC-3'.

Generation of transgenic plants

The JAG::VENUS vector was generated by first PCR-amplifying a 4.6 kb genomic fragment containing the JAG promoter and coding sequences from Ler genomic DNA with the primers T26Bl 5'-GAGATCCGGGAATAGCTGATGATGCCGTG-3' and T26Nr 5'-CCATGGCGAGCGATGATGATCTGTAACCCGATTGA-3'. The gene was translationally fused to the VENUS coding sequence separated by a 10-analine linker sequence in the transformation vector pMLBART (Gleave, 1992). The 35S::JAG vector was constructed by placing the JAG cDNA downstream of a Cauliflower Mosaic Virus (CaMV) 35S promoter in the binary vector pCGN1547 (McBride and Summerfelt, 1990).

The pOpL two-component system (Moore et al., 1998) was also used to misexpress JAG. A 6XOP::JAG expression vector was generated by first cloning the JAG cDNA downstream of six copies of the Operator sequence and an OCS minimal promoter in a modified version of the plasmid BJ36 (Gleave, 1992) a gift from Jeff Long (Salk Institute). The final expression cassette was in the transformation vector pART27 (Gleave, 1992). The 6XOP::JAG construct was transformed into a transgenic driver line pAPI: :LHG4 donated by John Bowman (University of California, Davis).

In situ hybridization

In situ hybridization was performed using digoxigenin-labeled RNA probes according to a published protocol (Long and Barton, 1998). The JAG antisense probe corresponds to a 500 bp 3'-specific cDNA sequence from plasmid pJAG0.5 that was linearized with BanHI and transcribed with T7 RNA polymerase, or a 1.0 kb 5'-specific cDNA from plasmid pJAG1.0 and similarly transcribed.

Results

The jagged mutation affects floral organ morphology

The Arabidopsis flower consists of four concentric whorls of organs that develop with distinct organ identity, position, number and size (Fig. 1A). Two ethylmethane sulphonate (EMS)-induced mutant alleles of the JAG locus were isolated in a genetic screen to identify genes that regulate floral organ morphology. The jag-2 and jag-3 alleles form a complementation group and overall display a similar recessive floral phenotype (Fig. 1C,D). The mutant perianth organs, sepals, petals, and stamens are narrow and ‘shredded’ in appearance, often with prominent jagged serrations at the distal tip of the sepal (Fig. 1B-E). The morphology of the jag-3 stage 13 petal is characterized by adaxial curling along the margins, as well as abnormal buckling along the lateral margins of the organ (Fig. 1F) that may be an indication of uncoordinated cell growth (cell division and/or expansion). Mature jag petal blades also appear slightly greenish in color compared to wild-type petals. The filaments of adjacent stamens are occasionally fused along the margins and the anther is variably reduced in size and altered in shape with a pointed distal tip (Fig. 1G and data not shown). The mature jag carpel valves appear to bulge with seed as if there was insufficient growth of the valves as seed developed within the siliques (data not shown) and the distal stigma appears to be incompletely fused (Fig. 1H).

Scanning electron microscopy (SEM) reveals that the coordinated outgrowth of floral organ primordia is disrupted in
**Fig. 1.** Morphology of jag floral and vegetative organs. (A) Wild-type Landsberg erecta flower. (B,C) jag-3 flower with narrow petals curling towards adaxial surface. Sepal with serrations at the distal tip is marked with an asterisk. (D) jag-2 flower. (E) SEM of jag-3 flower with a narrow sepal (arrowhead). (F) jag-3 abaxial petal with buckled margins. (G) jag-3 stamens fused along filament margins. (H) jag-3 gynoecium abnormally fused at the stigma. (I) Wild-type inflorescence with sepals enclosing floral buds. (J) jag-3 inflorescence with gynoecium protruding from floral bud (arrowhead). A wide medial sepal is present on the same flower (white arrow). A black arrow indicates a stage 7 flower with carpels protruding. (K) jag-3 early floral primordia with an extra sepal primordium initiated (arrow). (L) Petal primordium (p) from stage 9 jag-3 flower. (M) Petal primordium (p) from stage 10 jag-3 flower. (N) Wild-type 7-day post germination seedling. (O) jag-3 seedling with altered cotyledon shape. (P) Fifth rosette leaf from wild-type (left) or jag-3 (right) that has serrated margins. Scale bar: 100 μm (E-K,M); 10 μm (L); 1 mm (N-P).

**Table 1. Floral organ numbers in jagged mutant alleles**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of sepals*</th>
<th>Number of petals</th>
<th>Number of stamens</th>
<th>Number of carpels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>4±0</td>
<td>4±0</td>
<td>5.85±0.03</td>
<td>2±0</td>
</tr>
<tr>
<td>jag-2</td>
<td>4.21±0.04</td>
<td>4.16±0.04</td>
<td>5.73±0.04</td>
<td>2±0</td>
</tr>
<tr>
<td>jag-3</td>
<td>4.48±0.06</td>
<td>4.47±0.06</td>
<td>5.95±0.04</td>
<td>2±0</td>
</tr>
</tbody>
</table>

The first 10 flowers were examined from 12 individual plants of each genotype.

Values are the mean±s.e.m. of the number of organs per whorl.

*The number of sepals completely unfused along the margins was counted.

**jag mutants.** jag-3 mutant flower buds open prematurely (Fig. 1I,J) at floral stage 7, most likely because of more rapid proximal-distal outgrowth of the carpel primordia relative to the sepal primordia that normally enclose the inner whorls of reproductive organs (Fig. 1I). Both jag alleles cause a slight increase in the number of sepals and petals per flower, but the third and fourth whorl organs are similar in number to the wild type (Table 1). In contrast to the single sepal present at the adaxial position of the outer first whorl in wild type, the corresponding position in jag-3 flowers often contains either two sepals or a single wide sepal with two evenly spaced and distinct distal tips (Fig. 1D) that presumably represent either the congenital or postgenital fusion of two adjacent sepal primordia (Fig. 1I,K). Correlating with the development of extra sepals in the adaxial medial positions, an extra petal usually arises at the adaxial medial position in between the two adaxial sepals, thus giving five petals. Developing jag-3 petal primordia are abnormal in overall shape (Fig. 1L,M) compared to wild type (Smyth et al., 1990). Specifically the distal tip of the jag-3 petal primordium is irregular in shape and the proximal base is broadened rather than stalked.

**jag mutants also displayed minor defects in vegetative organs.** jag-3 cotyledon shape is abnormal (Fig. 1O) with the petiole and blade regions more elongated than the wild-type organ (Fig. 1N). jag mutant rosette leaves often have mild serrations along the lateral margins, in contrast to wild type (Fig. 1P).

**jag petals show defects in cell number and shape**

To further characterize the jag perianth organ defects, petals were examined from equivalent mature anthesis stage flowers of wild-type Landsberg erecta, jag-2 and jag-3 mutants. For the comparison of the abaxial epidermal surfaces of the wild-type and jag-2 mutant petals, the blade epidermis was equally subdivided along the proximal-distal axis into proximal, middle and distal regions containing distinct patterns of cell shape and size. The distal region in the wild-type petal contains small round epidermal cells with distinct cuticular ridges (Fig. 2A), while larger elongated cobblestone-shaped cells make up the proximal region (Fig. 2C). In the middle region of the wild-type blade, the epidermis is composed of a mixture of small round cells and cells that are both elongated in the proximal-distal axis and are intermediate in character to cells found in both the distal and proximal regions (Fig. 2B).

Adaxial epidermal cells in jag-2 petals show the characteristic wrinkled cuticle pattern of wild-type cells (Fig. 2A,D), indicating that petal organ identity is unaffected in the jag mutants. However in jag-2 mutants, there are no small round epidermal cells as in the distal region of the wild-type petal (compare Fig. 2A,D). Instead, cells located in the distal and middle regions of jag-2 petals (Fig. 2D,E) are elongated and cobblestone-shaped and resemble cells in the proximal regions of wild-type petals (Fig. 2C). The proximal region in jag-2 mutant petals contains larger abaxial epidermal cells that...
are more elongated than the corresponding cells in the wild-type petal (compare Fig. 2F and 2C). Because the margins of the jag mutant petals curl adaxially, it was difficult to flatten the organ to count the number of cells in the entire petal. Cell numbers are clearly reduced along the proximal-distal axis in the mutant (Fig. 2G,I) because the mutant epidermal cells are larger than the wild type ones. Cell numbers on the adaxial epidermal surface of the petals were similarly affected (data not shown).

The petals of jag-3 (Fig. 2H) showed a phenotype that was weaker than that of jag-2 (Fig. 2I), but distinguishable from wild type (Fig. 2G). Overall, the cells in the jag-3 petal epidermis were not as elongated as in jag-2. The distal abaxial epidermal region of the jag-3 petals contained a mixture of cells that were either round like wild-type distal epidermal cells, or elongated similar to those found in the jag-2 mutant.

In summary, jag mutant organs are narrower and somewhat irregular in shape compared to wild-type organs. This phenotype appears to result from defects in both cell division and cell expansion from early stages in their development. Examination of jag petals reveals that there are fewer epidermal cells than in the wild type but that these cells are larger than normal. This in turn suggests that jag cells are not restricted in their ability to expand but instead are unable to divide as rapidly.

**jag double mutants with genes involved in lateral organ development**

The phenotype of jag mutants closely resembles that of the antegumenta (ant) mutant. ANT regulates growth and cell division in floral organ primordia and ovules (Elliott et al., 1996; Klucher et al., 1996). To determine if there could be an overlap in the function of these two genes, double mutant plants were generated with the strong loss-of-function ant-9 allele. jag-3 ant-9 double mutant plants display a phenotype that is much more severe than either single mutant in that floral organs become reduced narrow structures that fail to elongate to the same extent as in either single mutant (Fig. 3A,B). This enhanced phenotype suggests that both ANT and JAG may function in parallel pathways that contribute to floral organ size and shape.

The filamentous flower (fil) mutant also produces narrow perianth organs (Fig. 3C) (Chen et al., 1999; Kumaran et al., 1999; Sawa et al., 1999). FIL is a key regulator of adaxial/abaxial patterning in lateral organs and functions redundantly with other members of the YABBY gene family (Siegfried et al., 1999). Double mutants between jag-3 and fil-1, a strong fil allele, have an additive floral phenotype (Fig. 3C,D). Numerous filamentous organs are produced in the place of petals and stamens in the double mutants. jag-3 fil-1 double mutant flowers have slightly narrower sepals in the first whorl compared to the single mutants, and in the fourth whorl the gynoecium has an elongated stylar region and a reduced carpel valve. These gynoecium abnormalities are observed in fil mutants and have been interpreted as a defect in proximal-
distal patterning in the floral primordium (Chen et al., 1999). Thus, the overall additive phenotype indicates that JAG acts independently of FIL and is therefore unlikely to function in patterning adaxial/abaxial organ polarity.

The SUPERMAN (SUP) gene is involved in regulating stamen and carpel development and floral organ number, possibly via the control of cell proliferation within the flower (Bowman et al., 1992; Sakai et al., 1995). Double mutant flowers harboring the null sup-5 allele and jag-3 displayed a completely additive phenotype characterized by jag-3 petal and sepal morphology, and increased stamen number and unfused carpel tissues similar to that observed in the sup-5 single mutant phenotype (Fig. 3E,F). These data indicate that JAG and SUP function independently in the processes of organ growth and organ number determination in the flower. clvata3 (clv3) mutants exhibit enlarged meristems and increased organ number in all floral whorls (Brand et al., 2000; Fletcher et al., 1999). clv3 jag double mutants also show an additive phenotype (data not shown) suggesting that JAG does not interact with other meristem genes of the CLAVATA signaling pathway involved in maintenance of cell proliferation in the shoot and floral meristem.

**JAG is required for bract outgrowth in leafy, apetala1 and apetala2 mutants**

The leafy (lfy) mutant produces flowers with only leaf-like organs, and bracts or floral leaves are produced at the base of each pedicel/peduncle (Fig. 4A) (Weigel et al., 1992). In lfy-6 jag-3 double mutants, the bract-like organs that develop at the base of the floral pedicel/peduncle are much reduced compared to lfy-6 single mutants and are frequently filamentous (Fig. 4B-D). The partial loss-of-function lfy-5 allele results in a less severe phenotype in the flower compared to lfy-6 that consists of bract-like sepals in the outer whorl and a slight reduction in petal and stamen formation in the inner whorls. Flowers mutant for both lfy-5 and jag-3 display characteristics associated with both single mutant, but the bract-like sepals in the lateral positions fail to grow out (compare Fig. 4E and F) and are often replaced with small projections. Occasionally, a few flowers lack outgrowth of all four bract-like sepals (data not shown). Flowers of lfy-6 jag-3 double mutants also appeared to produce fewer organs than the total number of floral organs produced in either single mutant, and they are arranged in a spiral pattern (data not shown). Thus, JAG is required for bract outgrowth both at the base of pedicels/peduncles and in the flowers in the lfy-5 jag-3 mutant background.

Bract-like sepals are also formed in the first whorl of 'A function' floral organ identity mutants such as ap1-1 and ap2-1 (Bowman et al., 1993; Bowman et al., 1991). To determine if JAG is also necessary for bract development in the flower, double mutant combinations were made. The ap1-1 jag-3 double mutant flowers produced fewer first whorl bract-like sepals than ap1-1 single mutants (Fig. 4J) in the early arising flowers after the transition to reproductive development. Similarly, the bract-like sepals of ap2-1 mutants failed to grow out, usually in the lateral positions, in ap2-1 jag-3 double mutant flowers (Fig. 4G,H), but sometimes all four first whorl organs failed to grow out (data not shown).

In summary, JAG is necessary for normal outgrowth of floral bract-like organs in the first whorl of lfy-5, ap1-1 and ap2-1 flowers, in addition to having a role in outgrowth of all four floral organs in the wild-type flower.

**Positional Cloning of JAG**

The position of the JAG locus was determined by its genetic linkage to BAC-specific molecular markers corresponding to Landsberg erecta and Columbia ecotype sequence polymorphisms using a recombinant F2 mapping population representing 856 chromosomes (Fig. 5A). Fine-scale mapping analysis revealed that JAG is located on BAC clone T26J14. Two independent overlapping 10 kb genomic fragments corresponding to BAC T26J14 sequences when transformed into plants rescued the jag-3 mutant phenotype (data not shown). Sequencing of candidate genes encoded by this genomic region in the jag-2 and jag-3 mutants revealed nucleotide sequence changes in the gene At1g68480. The JAG gene sequence was defined by genetic complementation to encompass a 4.5 kb genomic fragment and the coding exons were found to be interrupted by 5 introns. A cDNA sequence 1177 base pairs (bp) in length that is in agreement with a 1.2 kb transcript detected by northern blot (data not shown) was obtained from an inflorescence cDNA population.

The JAG locus encodes a 248 amino acid protein with motifs that suggest a role as a transcription factor (Fig. 5B). JAG belongs to a large family of C2H2-type zinc finger transcription factors that contains 112 members in Arabidopsis (Riechmann, 2002). JAG contains a single 31 amino acid single C2H2-type zinc finger motif (amino acid positions 51-81) that may have a function in...
Fig. 5. Map-based cloning and sequence of JAG. (A) Schematic of positional cloning of the JAG locus showing the frequency of recombinant chromosomes identified in a mapping population with respect to molecular markers corresponding to BAC clones. Fine-scale mapping localized the JAG locus to the BAC T26J14 (black box). (B) Amino acid sequence of the JAG protein. Positions and nature of the jag-2 (asterisk) and jag-3 (black arrowhead) mutations are indicated. The EAR repression motif is boxed. A putative nuclear localization sequence at position 35-38 and a short nine amino acid ERF-ear repression consensus motif is indicated below. Identical residues are in black boxes; similar residues in gray boxes. (C) Amino acid alignment of the JAG EAR motif with other Arabidopsis proteins. The positions of conserved cysteine and histidine residues involved in coordinating a zinc ion are indicated by asterisks. (D) Amino acid alignment of the JAG EAR repression motif with other Arabidopsis proteins. The EAR repression consensus motif is indicated below. Identical residues are in black boxes; similar residues in gray boxes.

DNA binding. A BlastP sequence search and alignment of the JAG zinc finger motif with other related Arabidopsis proteins reveals that JAG is closely related in sequence to the protein At1g13400 or JAG-like (29/31 identical amino acids in the zinc finger) while the proteins At5g05120, At1g67030 and SUPERMAN (SUP), have 19/31, 16/31 and 16/31 identical amino acids in the zinc finger, respectively, and are less closely related (Fig. 5C). All plant C3H2-type zinc finger proteins, including JAG, contain a conserved QALGGH sequence that has been shown in the SUP zinc finger to form part of an alpha-helix that contacts the major groove of DNA (Isernia et al., 2003).

Other motifs identifiable in the amino terminus of the JAG protein sequence include a putative nuclear localization sequence at position 35-38 and a short nine amino acid ERF-associated amphiphilic repression (EAR) motif (position 8-16) (Ohta et al., 2001). The repressor activity of the EAR motif has been demonstrated for the plant transcription factors ERF4 and JAT11 (Ohta et al., 2001) and for SUP where it has been shown to have a repressor function in vivo (Hiratsu et al., 2002). The JAG EAR motif contains three out of nine leucine residues and shows amino acid sequence conservation in an alignment with similar motifs present in previously characterized zinc finger transcription factors (Fig. 5D). Similarly to the SUP protein, JAG contains a proline-rich motif located at amino acids 129-141 in its carboxyl terminus that may function in transcriptional repression or activation. Taken together, the presence of these functional domains suggests that JAG is a nuclear-localized repressor of transcription.

The jag-2 mutation results in a premature stop codon at residue 54 at the start of the zinc finger motif, while the jag-3 mutation disrupts the 3'-splice acceptor sequence in the fourth intron, resulting in the production of a slightly larger transcript on northern blots (data not shown) and a predicted protein that is truncated at residue 104 with the addition of 11 extra amino acids. Consistent with the identified DNA lesions, the jag-2 allele is phenotypically slightly more severe than the jag-3 allele, according to our analysis of abaxial petal epidermis, and represents a stronger loss-of-function allele.

**JAG is expressed in lateral organ primordia and encodes a nuclear localized protein**

The developmental pattern of JAG mRNA localization was determined by in situ hybridization. JAG mRNA is first detected in late transition stage embryos in cells corresponding to the cotyledon primordia. JAG transcripts are uniformly localized throughout the newly emerging cotyledon primordia but are absent from the SAM (Fig. 6A). As the cotyledon primordia continue to grow out during the heart stage, JAG mRNA expression is excluded from the tips of the cotyledon primordia but continues to be expressed in a band below this region of repressed expression (Fig. 6B). In the torpedo stage embryo, JAG expression continues to be absent at the tip of the cotyledon primordia (Fig. 6C). Cells in the epidermis of the cotyledon have a lower level of JAG transcripts than cells in the inner layers. JAG is also expressed in developing leaf primordia of the seedling at 3.5 days and until 7 days after germination. It is initially expressed throughout the young primordium but later expression becomes excluded from the distal tip of the primordium (Fig. 6D-F). Reduced JAG expression is also detected at the proximal base of the leaf primordia (Fig. 6F). After the transition to reproductive development, JAG transcripts are absent in the inflorescence meristem but are detected in developing floral organ primordia (Fig. 6G). During flower development JAG transcripts are first detected in stage 3 flowers in sepal anlagen (Fig. 6H). Expression in the medial sepal occurs at the lateral sepals that are later to develop (Fig. 6H) and data not shown). A lower level of transcripts is detected at the distal tip of the sepal primordia of stage 4 flowers and onwards (Fig. 6G). Expression in the sepals decreases in stage 6-8 flowers and transcripts are detected in stamen and petal primordia, but expression is slightly reduced at the base of these primordia (Fig. 6G,H). Some JAG transcripts are detected in stage 8 carpel primordia (not shown) as well as in carpel valves in transverse sections of stage 8 gynoecia (Fig. 6L).
JAGGED promotes leaf growth

since the lfy-6 jag-3 double mutant phenotype indicated that JAG activity is important for lfy bract outgrowth. We found that in lfy-6 inflorescences JAG expression is detected in bract primordia (Fig. 6M) however, we were unable to detect transcripts in a domain within the inflorescence meristem corresponding to a bract anlagen.

To determine the subcellular localization of the JAG protein, a 4.5 kb genomic fragment containing putative 5' regulatory sequences plus the JAG coding sequences was translationally fused to VENUS, a rapidly maturing variant of Yellow Fluorescent Protein (Nagai et al., 2002). The transgene was expressed in jag-3 plants and completely rescued the mutant phenotype in 15 out of 17 T1 plants, indicating that the fusion protein was functional in plants (data not shown). The expression of the fusion protein mimicked the JAG RNA localization pattern as observed by in situ hybridization. Confocal laser scanning microscopy of a seedling at 4 days after germination shows the JAG-VENUS protein within the leaf primordium in a distinct band of expression (Fig. 6N). In leaf primordium cells, the JAG-VENUS protein co-localizes with Hoechst 33342 nuclear staining (Fig. 6O-Q). Consistent with the predicted protein function and the presence of a consensus nuclear localization sequence (Fig. 5B), the JAG-VENUS fusion protein is located in the nucleus.

In summary, JAG transcripts are exclusively localized to all lateral organ primordia throughout the plant, where expression is at first uniform throughout the young emerging primordia and then absent from the distal tips of organ outgrowth.

**JAG misexpression is sufficient for development of leaf-like tissues**

Since JAG is necessary to promote lateral organ growth, we tested whether JAG activity is sufficient for such growth by expressing it constitutively. A 35S::JAG construct expressed in both wild-type and jag-2 mutant backgrounds resulted in similar phenotypes. Detailed analysis of transgenic lines is described in the wild-type background. 35S::JAG transgenic plants showed disrupted organogenesis and the formation of ectopic leaf-like tissues throughout the plant.

The abnormal phenotype was first noted at the seedling stage. 35S::JAG hypocotyls often appeared green in color, in contrast to wild-type hypocotyls that are yellowish and lack high levels of chlorophyll (Fig. 7A). In addition, varying degrees of cotyledon fusion (44 out of 104 T1 plants) were observed, including either a single fused cotyledon or a goblet-shaped structure resulting from fusion along both lateral cotyledon margins (Fig. 7A). These seedlings were cleared using chloral hydrate to allow examination of the vasculature. In the wild-type cotyledons, the vasculature consists of a central midvein that runs along the proximal-distal axis and a small number of secondary veins that branch off from it (Fig. 7B). 35S::JAG cotyledons exhibit a highly irregular
vasculature in which a central midvein is not identifiable and the strands do not form a closed circuit (Fig. 7C). Many 35S::JAG seedlings also fail to develop leaves after germination. In those that do, the leaves are often irregular in shape and adjacent leaves may be fused along the petiole margins (Fig. 7D). Fusion of rosette leaves (arrow) and cotyledon (c) fusion along the petiole margins.

For more information, refer to the figure captions, which provide additional details about the images and the specific regions of interest. The captions are labeled from A to Q, each corresponding to a different figure. Scale bars vary in size, indicating different levels of magnification. The captions also mention various anatomical features such as midvein, petiole, and leaf margins, among others.
types found in leaves. Regions of the stem that lack visible leaf-like projections do however show abnormalities in the epidermis and cortex. The wild-type stem epidermis is composed of a single layer of small regular cells and the cortex is composed of about three cell layers of uniformly sized round cells (Fig. 8E). In contrast, the 3SS::JAG stem epidermal and cortical cells are variable in size and irregular in shape. (Fig. 8F).

Development of leaf-like tissues in place of flowers by JAG misexpression

Since the constitutive 3SS::JAG transgene resulted in a severe seedling phenotype that could obscure a floral gain-of-function phenotype, a two-component system (Moore et al., 1998) was used to express JAG under the control of the AP1 promoter. A driver transgenic line pAP1::LhG4 containing the AP1 promoter drives expression of the chimeric LhG4 transcriptional activator. When combined with an OP::GUS reporter gene in double transgenic plants, this pAP1::LhG4 driver results in a GUS staining pattern that mimics the AP1 expression pattern (data not shown). A 6XOP::JAG transgene was transformed into the pAP1::LhG4 driver line. 23 out of 25 T1 plants developed normally until the transition to flowering, when AP1 is first expressed throughout the stage 1 floral primordium (Mandel et al., 1992). Flower development was completely inhibited in pAP1::LhG4 6XOP::JAG plants. Instead leaf-like structures, pedicels with leaf-like outgrowths, or radially symmetric or club-shaped structures developed (Fig. 9A). Similar pedicels with leaf-like tissues were observed in 3SS::JAG plants (Fig. 7H.J). Although a cup-like receptacle structure formed at the distal end of some mature pAP1::LhG4 6XOP::JAG pedicels (Fig. 9B), no floral organs developed. We conclude that misexpression of JAG in the floral meristem is sufficient to promote growth of leaf-like tissues in the place of flowers, and thereby override normal floral organ development.

Discussion

JAG promotes growth of leaf tissues

Our study has uncovered a novel gene involved in lateral organ development. While JAG is not essential for the initiation of leaf primordia, it is required for the coordination of cell division and expansion during organ morphogenesis, and its expression, being limited to developing organ primordia, suggests that it functions directly in this process. However the jag phenotype is relatively mild compared to many other mutations affecting leaf development including fil/yabby (Siegfried et al., 1999), phab (McConnell and Barton, 1998) and pnh/zwille (Moussian et al., 1998; Lynn et al., 1999; Newman et al., 2002). It is therefore surprising that JAG misexpression appears capable of inducing the proliferation of leaf-like tissue in a variety of contexts. These observations suggest that perhaps the weak jag loss-of-function phenotype may be the result of genetic redundancy. One promising candidate is the JAG-like gene At1g13400 as it is the most closely related gene in the Arabidopsis genome and contains nearly identical C2H2 zinc finger and EAR repression motifs. Our preliminary analysis indicates that the expression pattern of At1g13400 overlaps with JAG in lateral organs and we are currently investigating loss-of-function double mutants (C.K.O. and E.M.M., unpublished).

The jag loss-of-function mutant shares some similarities in phenotype with two recently characterized mutants cincinnata (cin) and jaw. The Antirrhinum majus CIN gene encodes a TCP DNA binding factor that is involved in control of leaf shape and curvature by regulating cell cycle arrest in the distal region of the growing leaf (Nath et al., 2003). A similar leaf growth phenotype is associated with the activation-tagged mutant jaw. JAW encodes a microRNA similar in sequence to the Arabidopsis TCP4 gene and has been shown to negatively regulate TCP4 (Palatnik et al., 2003). Like jag petals, both cin and jaw mutant leaves are buckled along the margins. Furthermore, both TCP4 and JAG are normally expressed in

Fig. 8. Transverse sections of stem and leaf showing vegetative structure. (A) wild-type stem with central pith (p). (B) 3SS::JAG stem is larger in diameter than wild type with numerous finger-like projections and central pith (p). (C) Wild-type leaf showing upper and lower epidermis (arrowheads), palisade mesophyll (pm), spongy mesophyll (sm) and vascular bundle composed of phloem cells (black arrow) and xylem cells (white arrow). (D) Close-up image of finger-like projection of the 3SS::JAG stem with epidermis (arrowhead), phloem (black arrow) and xylem (white arrow). (E,F) Region of wild-type (E) and µm (B); 100 µm (C-F).
Fig. 9. Misexpression of JAG in flowers with pAP1:LhG4 6XOP::JAG. (A) Inflorescence with variable leaf-like organs in place of flowers. Organ are club-shaped with leaf-like pedicels after the transition to reproductive development. (B) A mature leaf-like organ. Scale bar: 100 μm.

JAG and bract formation

While bract formation is suppressed in Arabidopsis (Hempel and Feldman, 1994) and other members of the Brassicaceae family, a cryptic bract has been proposed to exist in Arabidopsis based on the expression of ANT in the inflorescence meristem as well as the complimentary expression of STM (Long and Barton, 2000). The expression of FIL and AS1 in a similar domain to that of ANT further supports this proposal (Byrne et al., 2000; Long and Barton, 2000; Siegfried et al., 1999). JAG is exceptional in this respect in that it is an early leaf development gene that is not expressed in the cryptic bract. Since constitutive expression of JAG is sufficient for the outgrowth of floral bracts, loss of JAG activity in the cryptic bract may be responsible in part for the absence of bracts in the Brassicaceae family. Consistent with this proposal, JAG is expressed in lfy mutant bracts and is required for their proper development.

A JAG-specific pathway of leaf tissue growth

Analysis of the phan mutant in Antirrhinum, led Waite and Hudson to propose that blade outgrowth requires the juxtaposition of adaxial and abaxial boundaries (Waite and Hudson, 1995; Waite et al., 1998). This model has been subsequently supported by studies of the organ polarity genes FIL, KAN1/KAN2, PHB, PHV, AGO and PNH/ZWILLE in Arabidopsis [summarized by Bowman et al. (Bowman et al., 2002)]. Given that JAG is the only gene known to be sufficient to promote growth of leaf tissue, it is important to determine whether JAG normally acts in response to such boundaries or whether it may promote boundary formation. In regard to this question it is worth noting that the JAG gain-of-function phenotype is sensitive to the context of the plant cellular environment, in that the phenotype produced by JAG and bract formation in any particular cell depends on the tissue in which it is expressed. For instance, although ectopic JAG expression results in regularly shaped floral bracts, the leaf-like tissue that develops from the stem appears disorganized and lacks any overall leaf-like shape. Hence patterning information appears to be largely absent although specific cell types associated with adaxial and abaxial leaf domains are present and are sometimes observed to be arranged in an organized manner. JAG expression, although occurring early in leaf development, also appears to be initiated slightly later than that of genes such as PHAB or FIL, supporting a role for JAG downstream of adaxial-abaxial boundary production. Although our genetic analysis of the fil-1 jag-3 double mutant suggests that JAG and FIL act in different pathways, in both cases genetic redundancy of JAG with JAG-like and FIL with YABBY3/YABBY2 (Siegfried et al., 1999) may mask the participation of the individual genes in organ growth and abaxial polarity specification. We therefore favor the proposal that JAG normally acts downstream of leaf patterning genes during lateral organ development, in order to promote the wild-type pattern of cell division and differentiation. It will be interesting to try to separate these JAG-specific functions by identifying the downstream targets of JAG gene activity.

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