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NUBBIN and *JAGGED* define stamen and carpel shape in *Arabidopsis*

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An error in Fig. 4 was not corrected before the article went to press.

Panel B should be labelled as wild type (WT) and panel C as *jag nub*. The legend should read:

Similar to wild type (A), *FIL* expression in stage 6 *jag nub* stamens (C) is observed throughout the abaxial half. Although the *FIL* expression domain is soon dwarfed by the proliferation of the microsporangia in wild type, in *jag nub* anthers, no microsporangia proliferation is apparent and the *FIL* expression domain extends towards the adaxial side (D).

The authors apologise to readers for this mistake.

NUBBIN and *JAGGED* define stamen and carpel shape in *Arabidopsis*

José R. Dinneny^{1,2}, Detlef Weigel^{2,3} and Martin F. Yanofsky^{1,*}

Differential growth of tissues during lateral organ development is essential for producing variation in shape and size. Previous studies have identified *JAGGED* (*JAG*), a gene that encodes a putative C₂H₂ zinc-finger transcription factor, as a key regulator of shape that promotes growth in lateral organs. Although *JAG* expression is detected in all floral organs, loss-of-function *jag* alleles have their strongest effects on sepal and petal development, suggesting that *JAG* may act redundantly with other factors in stamens and carpels. Here, we show that *NUBBIN* (*NUB*), a gene closely related to *JAG*, is responsible for this redundancy. Unlike *JAG*, *NUB* is exclusively expressed in leaves, stamens and carpels, and briefly in petal primordia. Furthermore, whereas *JAG* expression extends into all cell layers of lateral organs, *NUB* is restricted to the interior adaxial side. Our analysis focuses on stamen and gynoecium development, where we find that *NUB* acts redundantly with *JAG* to promote the growth of the pollen-bearing microsporangia of the anthers and the carpel walls of the gynoecium, which enclose the ovules. *JAG* and *NUB* also act redundantly to promote the differentiation of adaxial cell types in the carpel walls, and in the establishment of the correct number of cell layers. The important role these two factors play in regulating organ growth is further demonstrated by gain-of-function experiments showing that ectopic *NUB* expression is sufficient to drive the proliferation of tissues and the amplification of cell-layer number.

KEY WORDS: *Arabidopsis*, Development, Lateral organs, Growth, Patterning, Polarity, Floral organs

INTRODUCTION

As a result of evolution, lateral organs have assumed a vast array of shapes and sizes that adapt the sessile plant to diverse environments. In order for shape differences to emerge during organogenesis, morphogenesis must be carefully regulated by pathways that promote, maintain or suppress growth. Recently, several genes have been identified that play crucial roles in determining organ shape by regulating growth (Crawford et al., 2004; Dinneny et al., 2004; Ha et al., 2003; Hepworth et al., 2005; Nath et al., 2003; Norberg et al., 2005; Ohno et al., 2004; Palatnik et al., 2003). The C₂H₂ zinc-finger transcription factor encoded by *JAGGED* (*JAG*) is expressed in all organs and appears to act as a region-specific promoter of growth. In a loss-of-function *jag* background, lateral organ growth is partially suppressed. In petals, a correlation between the expression domain of *JAG* and the abundance of cells actively engaged in the cell-cycle suggests that *JAG* may regulate growth by maintaining or activating cell-cycle activity. Furthermore, the phenotypic similarity between *jag* loss-of-function mutants and transgenic lines in which cyclin-dependent kinase inhibitors are overexpressed, suppressing cell-cycle activity, further hints at such a connection (De Veylder et al., 2001; Wang et al., 2000). Unlike other genes that promote growth, such as *AINTEGUMENTA* and *BIG BROTHER* (Disch et al., 2006; Krizek, 1999; Mizukami and Fischer, 2000), *JAG* is also sufficient to promote morphogenesis in multiple types of lateral organs. In rosette leaves, for example, where *JAG* activity is normally limited to the blade region, ectopic expression can promote blade tissue expansion into the petiole region.

The ability of ectopic *JAG* activity to promote blade tissue led to the hypothesis that the *BLADE-ON-PETIOLE* (*BOP*) genes might negatively regulate *JAG* expression (Ohno et al., 2004). Like *JAG* gain-of-function lines, loss of *BOP* activity results in the ectopic development of blade tissue in the petiole region of leaves (Ha et al., 2003). Recent work characterizing the *bop1,2* double mutant has further elucidated the role of *BOP* genes in lateral organ development. The *BOP* genes negatively regulate *JAG* expression in lateral organs, particularly in the cryptic bract, a vestigial organ in *Arabidopsis* that subtends floral primordia, but which develops as a macroscopic leaf-like organ in other species (Hepworth et al., 2005; Norberg et al., 2005). Ectopic expression of *JAG* in the cryptic bract rescues organ outgrowth, whereas loss of *JAG* activity suppresses bract outgrowth in mutants that ectopically develop bracts (Dinneny et al., 2004; Ohno et al., 2004). However, when *JAG* activity is removed from *bop1,2* double mutants, which also develop ectopic bracts, no suppression of the *bop*-mutant phenotype was seen (Norberg et al., 2005). The observation that ectopic expression of a gene closely related to *JAG*, At1g13400 [previously referred to as *JAGGED-LIKE* and renamed *NUBBIN* (*NUB*) in this work], is also detected in *bop1,2* mutants suggests that other factors in addition to *JAG* may be responsible for the ectopic tissue growth in *bop* mutants (Norberg et al., 2005). Independently, we have hypothesized that *NUB* may function redundantly with *JAG* (Dinneny et al., 2004), because *JAG* does not equally affect all lateral organs in which it is expressed. Here, we have used gain-of-function and loss-of-function approaches to reveal the function of *NUB* in development. We focus our analysis on the role of *NUB* and *JAG* in stamen and carpel development, where *NUB* is expressed in a polar manner on the organ surface oriented toward the shoot axis, termed the adaxial side. The polar expression of *NUB* on the adaxial side correlates with the growth and patterning defects observed in *jag nub* mutants.

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MATERIALS AND METHODS

Plant material

Wild type was Col-0. *jag-1* (Dinneny et al., 2004) and *SPL::GUS* (Ito et al., 2004) have been described before. Seeds for the T-DNA insertion line 244A08 (also known as *nub-1*) were obtained from GABI-Kat (Rosso et al., 2003).

Genotyping

Genotyping of *jag-1* has been described before (Dinneny et al., 2004). To genotype *nub-1* we used primers oJD153 (AAG ACA GCG GAG GAT AAA GAT ATG) and oJD154 (GCT TCT CTT CAT CTT CTT CTT CTG G) to detect the wild-type allele and oJD155 (GTT CAT GTG GCC ACC GAG AGC TTG) and GABRB (GTG GAT TGA TGT GAT ATC TCC) to detect the *nub-1* allele.

Construction of transgenic plants

The *NUB-RNAi* construct was made by amplifying a 326 bp *NUB* cDNA fragment using primers N-1392 (ATA GCT TTC CTC CTC ATC AAG GAC) and N-1393 (ATG TGG AGC AAC TCT AGA ACC ATT A), and cloning this into the pHANNIBAL vector (Wesley et al., 2001) in forward and reverse orientations (pJD93). The *NUB-RNAi* cassette was then inserted 3' of an *APETALA1* promoter fragment derived from pAM571 (Mandel et al., 1992) using *Bam*HI restriction sites (pJD108). The *FIL::NUB* construct was made by amplifying a 4 kb *FILAMENTOUS FLOWER* promoter fragment, using primers oJD182 (gag ctc CAA CCA TTG AAC CAT CAC CGA TAT TC) and oJD183 (ggt acC TTT TTT GTA AGA AGG GGA AAA ATA TTG GAA GCT G) that introduced *Sac*I and *Kpn*I restriction sites (indicated by the lowercase letters) to the 5' and 3' ends, respectively. This fragment was then cloned into the pMX202 (Wu et al., 2003) binary vector (pJD136). A full-length *NUB* cDNA fragment was then amplified from pPY1 (Dinneny et al., 2004), using primers oJD211 (ggt acc ATG AGA GCT GAT GAA AAT AAC AC) and oJD212 (ggt acc TTA TAG CCC ATG ATG TGG AGG TAG ATG) that introduced *Kpn*I restriction sites to the 5' and 3' ends, and cloned into pJD136 (pJD148). Transgenic Col-0 plants were generated by the floral-dip method (Clough and Bent, 1998).

RT-PCR

RNA amplification and reverse transcription reactions were performed as previously described (Kardailsky et al., 1999). PCR amplification was performed on 2 μ l of reverse-transcription reaction. A *NUB* fragment encompassing the entire coding region was amplified using oJD119 (AAG CTT ATG AGA GCT GAT GAA AAT AAC AC) and oJD120 (GGA TCC TTA TAG CCC ATG ATG TGG AGG). A *TUBULIN* fragment was amplified as previously described (Dinneny et al., 2004).

In situ hybridization, histology and microscopy

In situ hybridization was performed as previously described (Dinneny et al., 2004). An anti-sense *NUB* probe was transcribed using T7 RNA polymerase (Promega) from a partial *NUB* cDNA clone (pJD109) linearized with *Hind*III. The *JAG* (Dinneny et al., 2004), *FIL* (Siegfried et al., 1999) and *PHB* (McConnell et al., 2001) probes have previously been described. Scanning electron microscopy (SEM) was performed as previously described (Dinneny et al., 2004). Embedding of plant material in JB-4 media, sectioning and staining with Toluidine Blue was performed as previously described (Roeder et al., 2003). Saffranin O and Alcian Blue staining of paraffin sections performed as previously described (Roeder et al., 2003). GUS staining performed as previously described (Blázquez et al., 1997).

Measurements of cell layer number and average cell width

Cell layer number and cell width measurements were determined from the same transverse cross-sections of stage-13 gynoecia. For each genotype, 16 sections were imaged from four gynoecia. Sections were taken from the approximate middle of the gynoecium and were separated by at least 30 μ m from each other. Cell layer number was measured in four regions of the carpel. Two counts were obtained from valve tissues flanking the main vascular bundle of the valve (lateral region) and two counts were obtained from valve tissues flanking the presumptive valve margins (medial region). The average width of cells in the *ena*, *enb* and outer epidermal cell layers

were determined as follows. First, the total width of all cells in a particular cell layer was measured using Image J software (<http://rsb.info.nih.gov/ij/>). Next, the number of cells in this layer was determined. The average cell width was then calculated by dividing the width of all cells in a layer by the number of cells in that layer. Both valves of the gynoecium in each section were counted separately.

Abaxial trichome number during leaf development was determined for the area of the leaf blade outside of the midvein, as this region normally contains trichomes in most leaves. Segregating T2 transgenic *FIL::NUB* plants were selected on MS agar plates containing 20 μ M kanamycin then transferred to soil. Wild-type control plants were also germinated on MS agar plates before being transferred to soil. Trichome counts were performed on plants after bolting. Kanamycin is unlikely to affect abaxial trichome development, as a *FIL::GFP* transgenic line germinated on kanamycin-supplemented agar plates did not develop ectopic abaxial trichomes (data not shown).

RESULTS

NUB is expressed on the adaxial side of lateral organs

In order to determine what roles *NUB* may play in development, we examined its expression in wild-type plants (Fig. 1). During vegetative development, *NUB* is expressed in initiating leaf primordia (Fig. 1A-C). Expression is restricted to the inner surface of primordia, termed the adaxial side. Unlike other genes in *Arabidopsis* expressed on the adaxial side of leaves, such as *PHABULOSA* (*PHB*), *PHAVOLUTA* and *REVOLUTA*, *NUB* is not expressed in the shoot meristem (Emery et al., 2003; McConnell et al., 2001; Waites et al., 1998).

Like *JAG*, *NUB* expression is excluded from the cryptic bract (Fig. 1D). Unlike *JAG*, however, *NUB* expression is also excluded from sepal primordia (Fig. 1D). The expression of *NUB* in petals is transient and can only be detected during the initial outgrowth of the primordia (Fig. 1K). During stamen development, *NUB* is strongly expressed throughout initiating primordia during stage 5 and then becomes restricted to the adaxial side by stage 6 (Fig. 1E-G). At stage 8, *NUB* expression becomes restricted to a small cleft of tissue in between the two sets of microsporangia (Fig. 1H). This expression pattern differs from *JAG*, which is expressed throughout stamen primordia (see Fig. S1 in the supplementary material). During carpel development, *NUB* is initially expressed in cell layers of both abaxial and adaxial halves (Fig. 1G), it then becomes restricted to the adaxial cell layers at stage 7 (Fig. 1H,I). *NUB* expression can also be seen on the adaxial side of initiating ovule primordia (Fig. 1J). In summary, *NUB* expression is more restricted than that of *JAG* and is found predominantly in leaf, stamen and carpel primordia. Furthermore, although *JAG* is expressed throughout all tissue layers of organ primordia, the adaxial expression of *NUB* suggests that it may play a role in promoting adaxial-specific development.

Identification of the *nub-1* allele

We identified a T-DNA insertion allele of *NUB*, 244A08, in the GABI-Kat collection (Rosso et al., 2003). Sequencing of the insertion site by GABI-Kat indicates that line 244A08 has a T-DNA disrupting the second intron of *NUB* (Fig. 2A). Examination of *NUB* transcript levels shows that the *nub-1* mutation strongly diminishes the accumulation of the *NUB* full-length transcript (Fig. 2B). The homozygous mutant does not display an abnormal phenotype (data not shown). This was not entirely unexpected, as the *NUB* expression domain is fully encompassed by the *JAG* expression domain. The *jag nub* double mutant, however, results in a strong enhancement of defects in leaf, stamen and carpel development compared with *jag* single mutants (see below). To ensure that these

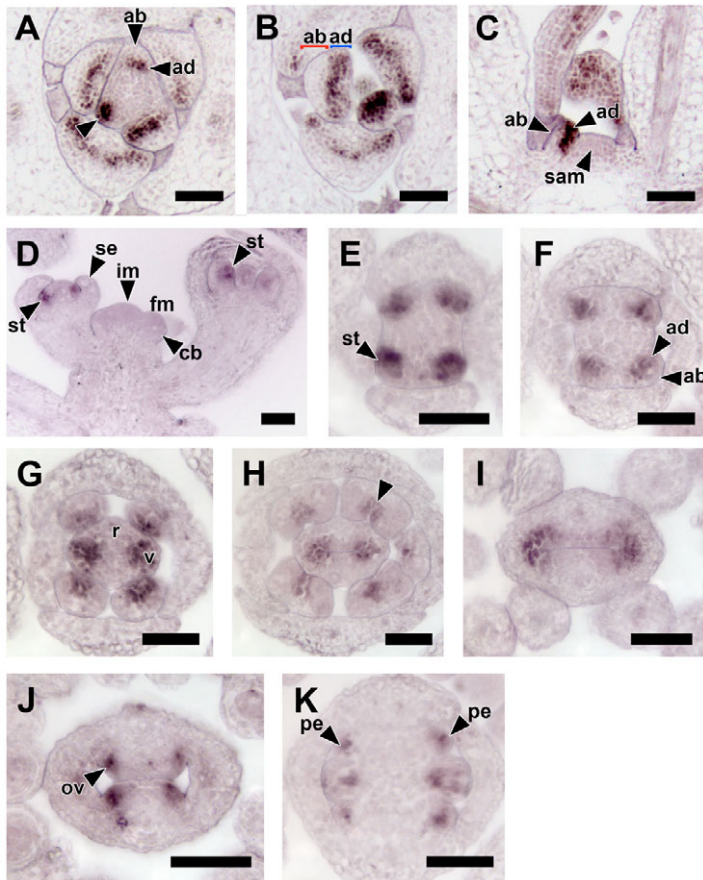


Fig. 1. *NUB* expression during lateral organ development. (A-K) *NUB* expression detected by in situ hybridization using an anti-sense probe on wild-type tissue. (A-C) Transverse (A,B) and longitudinal (C) cross sections of vegetative shoots. *NUB* expression starts in leaf primordia during initiation (A) and quickly becomes restricted to the adaxial side (B). *NUB* expression is not detected in the shoot apical meristem (C). (D) *NUB* expression is not detected in the inflorescence meristem, early stages of flower meristem development, cryptic bract or sepal primordia. (D-G) *NUB* expression in stamen primordia is apparent just prior to organ initiation, and soon becomes restricted to the adaxial side after initiation. (E) Stage 5 flower showing *NUB* expression throughout early stamen primordia. During late stage 5 (F) and stage 6 (G), *NUB* expression becomes restricted to the adaxial side of stamen primordia. (H) During stage 7, *NUB* expression becomes restricted to a group of cells in between the two sets of microsporangia (black arrowhead). (G) During gynoecium development, *NUB* is initially expressed in all cell layers of the valves at stage 6. During stage 7 (H,I), *NUB* expression becomes restricted to the adaxial side of the valves and is maintained there until stage 8 (not shown). (J) During early stage 9, *NUB* expression can be detected in ovule primordia. (K) *NUB* expression is briefly detected in petal primordia at stage 6. ab, abaxial; ad, adaxial; cb, cryptic bract; fm, floral meristem; im, inflorescence meristem; ov, ovule; pe, petal; r, replum; sam, shoot apical meristem; se, sepal; st, stamen; v, valve. Scale bars: 50 μ m.

enhanced defects are specifically due to the *nub-1* mutation, we generated a *NUB-RNAi* construct and introduced this into a *jag* mutant background and observed similar defects in stamens and carpels as in *jag nub* mutants (Fig. 2C,D). Sepal development is also affected indicating that our *NUB-RNAi* construct may result in some non-specific degradation of other transcripts (data not shown).

***NUB* acts redundantly with *JAG* to promote growth and patterning in stamens and carpels**

Examination of flower development in *jag nub* mutants reveals that the effect of the *nub* mutation is restricted to the center two whorls. Defects in sepal development observed in *jag* single mutants are not enhanced by *nub* (Fig. 3A-C). Petal growth also tends to be similar between *jag* single mutants and *jag nub* double mutants; however, the phenotype of *jag nub* petals is more consistent (data not shown), whereas defects in petal growth are more variable in *jag* flowers (Dinneny et al., 2004).

***NUB* promotes the growth of the adaxial side of anthers**

Stamens are divided into two regions, an apical anther and a basal filament (Bowman, 1993). In *jag nub* mutants, anther development is nearly abolished, whereas the filament does not appear to be affected (Fig. 3, see also Fig. S2 in the supplementary material). We examined the anther region more closely to determine whether any anther-specific cell types remained in *jag nub* mutants. On the adaxial side of wild-type anthers, four microsporangia develop, which enclose the developing pollen in chambers termed locules (Fig. 3D,K). During anthesis, the pollen is released from the anthers at a dehiscence zone that develops between the two locules in each

pair. On the abaxial side, a small lump of tissue, termed the connective, joins the two sets of microsporangia to the filament (Fig. 3G,K). When we examined the cells present in the stub of tissue that remains in the anther region of *jag nub* mutants, we saw that it is composed of two different domains (Fig. 3F). In the apical half of the stub, cells with a slight puzzle shape form with cuticular ridges (Fig. 3F,I). In the basal region, cells with a rounded shape develop without cuticular ridges (Fig. 3F). The apical cells resemble those normally found in the connective region of the anther (Fig. 3H), whereas the basal cells do not resemble any cell type found in the anther.

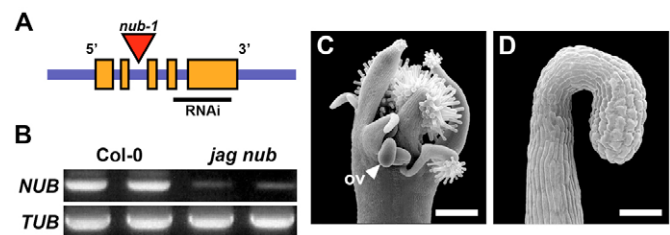


Fig. 2. The *nub-1* insertion allele. (A) Diagram of the *nub-1* T-DNA insertion allele. (B) Expression of *NUB* and *TUBULIN (TUB)* detected by reverse transcription followed by polymerase chain reaction (RT-PCR), using RNA isolated from Col-0 and *jag nub* inflorescence tissue. (C,D) Phenotypes observed in *AP1::NUB-RNAi jag-1* transgenic plants. (C) The valve tissues are often shredded near the apical region of the gynoecium, resulting in exposure of the ovules. (D) Microsporangia development is absent in the anther (compare with Fig. 3D). ov, ovule. Scale bars: 200 μ m in C; 50 μ m in D.

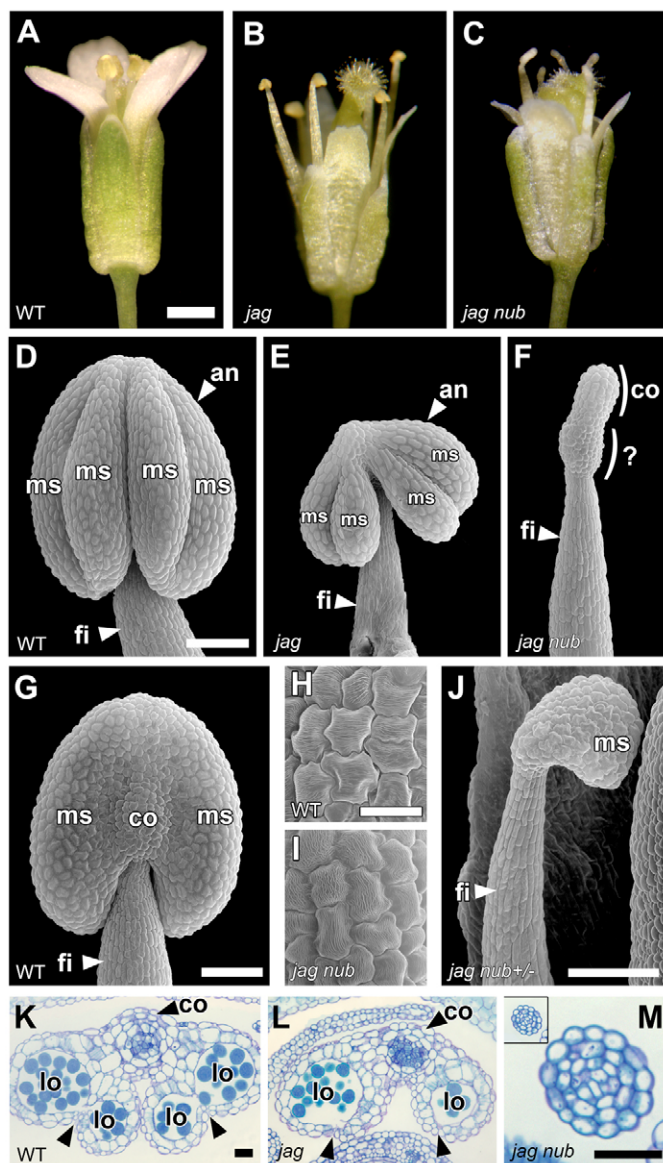


Fig. 3. Flower and anther development in *jag nub* mutants. SEM images are of stamens taken from stage 12 flowers. (A–C) Wild type (A), *jag* (B) and *jag nub* (C) flowers. Sepal and petal development is similar between *jag* and *jag nub* mutant flowers; however, anthers are reduced to stubs of tissue and the distal region of the *jag nub* gynoecium is abnormal. (D–F) SEM images of the adaxial side of anthers from wild-type (D), *jag* (E) and *jag nub* (F) stamens. Four elongated sacs of tissue (microsporangia) are apparent in wild type and *jag* mutants. In *jag nub* mutants, however, a small stub of tissue develops instead, with epidermal characteristics of the connective in the apical region and cells of uncertain identity in the basal region. (G) SEM image of the abaxial side of a wild-type anther revealing the connective that develops in between the microsporangia. (H,I) SEM images of epidermal cells taken from the connective region of wild-type anthers (H) and the connective-like region of *jag nub* anthers (I). (J) SEM image of a stamen from a *jag nub^{+/-}* mutant showing partial development of the microsporangia. (K–M) Plastic-thin sections (3 μm) of wild type (K), *jag* (L) and *jag nub* (M) anthers. (K) In wild-type anthers, four locules develop on the adaxial side separated by the connective on the abaxial side. Black arrowheads indicate dehiscence zones. (L) In *jag* mutants, two locules appear to develop and will split through a dehiscence zone that develops down the middle of the microsporangia (black arrowheads). (M) In *jag nub* mutants, no locule or pollen development is apparent. Inset is the same magnification as K and L. an, anther; co, connective; fi, filament; lo, locule; ms, microsporangia. Scale bars: 0.5 mm for A–C; 100 μm for D–G,J; 25 μm for H,I; 50 μm for K–M.

although the external structure suggests that four microsporangia have formed, the internal structure suggests that only two locules are patterned in *jag* mutants, or that the tissue that normally separates the locules does not develop properly.

FIL expression marks the connective tissue of the anther

To further characterize the defects in stamen development seen in *jag nub* mutants, we examined the expression of genes that mark different regions of the anther. We first examined *FIL*, which is initially expressed on the abaxial side of anthers (Fig. 4A). Later, as tissues on the adaxial side proliferate to form the microsporangia, *FIL* expression remains on the abaxial side in tissues that do not proliferate as much and become the connective (Fig. 4B). In *jag nub* mutants, *FIL* expression initiates in the anthers as it does in wild type (Fig. 4C). During stage 7, however, *FIL* expression extends toward the adaxial side (Fig. 4D). The apparent expansion of *FIL* onto the adaxial side of the anther may indicate that abaxial fate has expanded to the adaxial side in *jag nub* anthers. Alternatively, the expansion of *FIL* may be an indirect consequence of unequal growth of the abaxial side in relation to the adaxial side in *jag nub* mutants. In either case, these data suggest that the anthers of *jag nub* mutants are largely composed of connective tissue, marked by *FIL* expression, and that the adaxial microsporangia tissues fail to proliferate.

PHB expression marks the dehiscence zone of anthers

We next examined genes that are normally expressed in tissues on the adaxial side of the anther. *PHB* is initially expressed in the vasculature and on the adaxial side, but it soon becomes restricted to cells that denote the two dehiscence zones of the anther (Fig. 4E). In *jag nub* mutants, where no microsporangia development is apparent, *PHB* expression is limited to a few cells in the center of

We next examined anthers in plastic thin sections to determine the cell types that develop internally. Wild-type anthers develop four locules within the microsporangia (one locule per microsporangium) where the pollen grains form (Fig. 3K). In *jag nub* mutants, no such tissues can be found (Fig. 3M). These data show that locule development does not occur in *jag nub* mutants and that the stub of tissue that remains may mostly be composed of abaxial connective. We have also found that the effect of the *nub* mutation on anther development is semi-dominant when in a *jag* mutant background. In *jag nub^{+/-}* mutants, some microsporangia growth is still apparent (Fig. 3J). In addition, these plants are partially fertile, indicating that they are capable of producing pollen (data not shown).

We also took a closer look at the anthers of *jag* single mutants in plastic sections and found that there seemed to be fewer locules (Fig. 3L). This was at first surprising, as the anthers appeared to have four microsporangia when imaged by SEM (Fig. 3E). When the sections of *jag* anthers were examined more closely it could be seen that a narrow crease still develops and runs down the center of the microsporangia at the point which would normally divide the two locules and where the anthers normally dehisce (Fig. 3L). Thus,

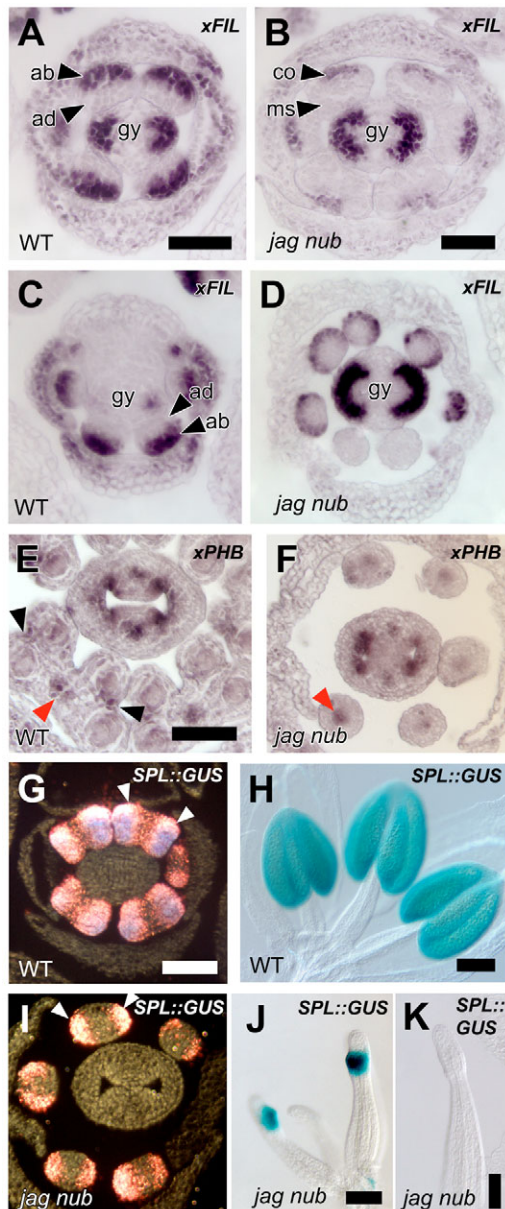


Fig. 4. Characterizing *jag nub* patterning defects in the anthers using *FIL*, *PHB* and *SPL* expression. (A–D) Expression of *FIL* detected by in situ hybridization using an anti-sense probe. (A) During wild-type stamen development, *FIL* is initially expressed throughout the abaxial side at stage 6. (B) As the adaxial microsporangia proliferate, *FIL*-expressing tissues develop into the connective and grow more slowly in comparison to the adaxial region. Similar to wild type (C), *FIL* expression in stage 6 *jag nub* stamens (D) is observed throughout the abaxial half. Although the *FIL* expression domain is soon dwarfed by the proliferation of the microsporangia in wild type, in *jag nub* anthers, no microsporangia proliferation is apparent and the *FIL* expression domain extends towards the adaxial side. (E,F) Expression of *PHB* detected by in situ hybridization using an anti-sense probe. (E) In wild type, *PHB* expression is detected in cells marking the dehiscence zone in between the two microsporangia in each pair (black arrowhead), as well as in the vasculature (red arrowhead). (F) In *jag nub* mutants, *PHB* expression is only detected in the vasculature. (G–K) *SPL* expression monitored using the *SPL::GUS* reporter. In wild type, *SPL*-reporter activity marks two domains in the anther that develop into the two sets of microsporangia (G, stage 6 shown, white arrowheads) and is maintained in the anthers until anthesis (H, stage 12 shown). (I) In *jag nub* mutants, the *SPL* reporter is activated in a similar spatial pattern to wild type. *SPL*-reporter activity is maintained in *jag nub* anthers until about stage 11 (J), but then disappears by anthesis (K, stage 12 shown). ab, abaxial side; ad, adaxial side; co, connective; gy, gynoecium; ms, microsporangia. Scale bars: 50 μm.

the *jag nub* anther, presumably marking the vasculature (Fig. 4F). Note that *PHB* expression on the adaxial side of the carpels is not affected in *jag nub* mutants (Fig. 4E,F).

***SPOROCTELESS*, a marker of microsporangia development is initially expressed in *jag nub* anthers**

To further explore why the adaxial microsporangia region of the anther does not properly develop in *jag nub* mutants, we examined the expression of *SPOROCTELESS* (also known as *NOZZLE*) using an *SPL::GUS* reporter line that marks cells of the microsporangia (Ito et al., 2004; Schiefthaler et al., 1999; Yang et al., 1999). During wild-type anther development, the *SPL* reporter is expressed in two domains on the peripheral flanks of the anther (Fig. 4G) (Ito et al., 2004). Surprisingly, in *jag nub* mutants, where microsporangia formation is not apparent, *SPL* reporter activity is still initiated in the proper domains (Fig. 4I). Later during anther development, *SPL* reporter activity is localized to the basal region of *jag nub* anthers, where we observed rounded

cells with little differentiation (Fig. 4J). Finally, reporter activity is lost in the anther region of *jag nub* mutants (Fig. 4K), whereas in wild type, expression is maintained until anthesis (Fig. 4H). These data show that, although microsporangia development is blocked in *jag nub* mutants, the patterning of the microsporangia domains appears to occur normally. Thus, *JAG* and *NUB* may not be required to specify microsporangia per se, but instead act in parallel to such specification pathways to promote the growth of these tissues. This function of *JAG* and *NUB* may be similar to the role that *JAG* plays in petal development where it promotes distal petal growth.

Abaxial *NUB* expression promotes connective growth

To further test whether *NUB* regulates microsporangia identity or plays a more restricted role in promoting the growth of this region, we used the *FIL* promoter to drive *NUB* expression on the abaxial side of anthers in the connective region. We reasoned that if *NUB* is sufficient to promote microsporangia identity, then *FIL::NUB* transgenic lines might be expected to promote ectopic microsporangia formation in place of the connective. Alternatively, if *NUB* regulates growth, but does not control the identity of microsporangia tissues, then we might expect the connective to become enlarged in *FIL::NUB* anthers but still retain the same cell types as in a wild-type connective.

As shown in Fig. 5, introduction of a *FIL::NUB* transgene into wild-type plants results in anthers with expanded abaxial tissue. In moderately affected lines, such as JD148-B, most of the ectopic growth is localized to the connective region, resulting in an enlarged and elongated structure (Fig. 5A,B). In stronger lines, such as JD148-A, the entire abaxial side appears to become overgrown and can be seen exceeding the limits of the adaxial microsporangia (Fig. 5C,D). To determine whether this ectopic

tissue is composed of microsporangia-like or connective-like cell types, we analyzed plastic thin sections of *FIL::NUB* anthers and found it to be composed of cells most similar to the connective (Fig. 3K, Fig. 5E).

The observation that *FIL::NUB* transgenic lines have an abnormal phenotype suggests that the *FIL* promoter is active in these lines. We confirmed our prediction by examining *FIL* expression in *FIL::NUB* flowers and found *FIL* to be expressed on the abaxial side in two cell layers (Fig. 5F). Interestingly, the expression of *FIL* in anthers appeared to have expanded laterally, probably because of the positive effect of *NUB* expression on tissue proliferation. Thus,

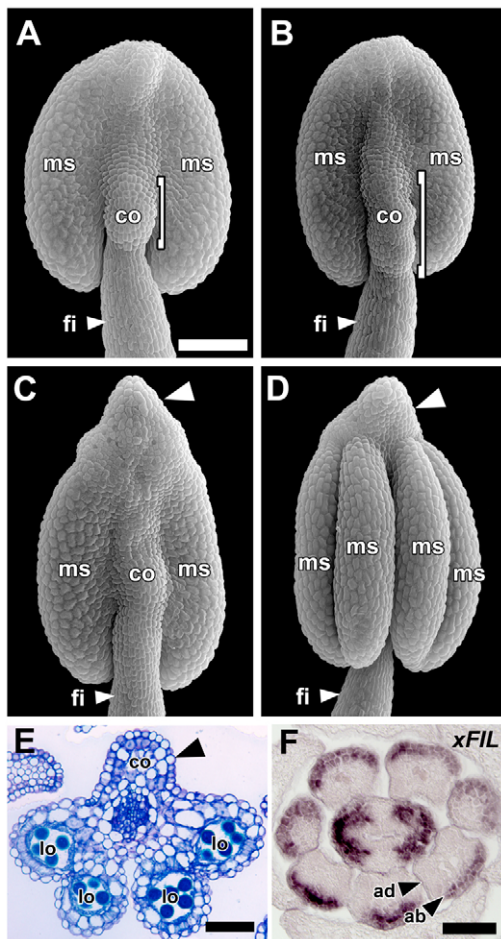


Fig. 5. Abaxial *NUB* expression promotes connective growth.

(A-D) SEM images of the abaxial side (A-C) and adaxial side (D) of anthers from plants transformed with a *FIL::NUB* transgene. (A,B) The JD148-B transgenic line develops moderate defects in anther morphology. Anthers from this line develop enlarged connectives (A) that can also be elongated (B). (C,D) The JD148-A transgenic line develops strong defects in anther morphology. Anthers from this line develop enlarged and elongated connectives, as well as extended sheets of abaxial tissue that extend apically beyond the normal limits of the anther (white arrowhead). (E) Plastic-thin section through a stage 10 anther from a *FIL::NUB* transgenic line that develops strong defects in anther development. Ectopic tissue proliferation can be seen in the connective region (black arrowhead). No pollen development occurs in the ectopic tissue, indicating that this tissue does not have microsporangia identity. (F) *FIL* expression detected by in situ hybridization using an anti-sense probe. *FIL* expression can be seen in this stage 7 flower throughout the abaxial side. ad, adaxial; ab, abaxial; co, connective; fi, filament; lo, locules; ms, microsporangia. Scale bars: 50 μ m.

despite being normally expressed in mutually exclusive domains, *FIL* expression is compatible with *NUB* expression in *FIL::NUB* transgenic lines. These findings differentiate *NUB* from other adaxially expressed genes such as *PHB*, which are sufficient to suppress *FIL* expression if mis-expressed on the abaxial side (Siegfried et al., 1999).

***NUB* promotes the growth of the valves**

Although the gynoecium is mildly affected in *jag* single mutants (Dinnyen et al., 2004; Ohno et al., 2004), *jag nub* double mutants show a strong decrease in the growth of the valves near the apical region of the gynoecium, resulting in exposure of the ovules (Fig. 6G,H). Tissues not associated with the valves, such as the replum and style seem to be less affected and grow apically beyond the limits of the valves. The splitting of the style, which was occasionally observed in *jag* mutants, is further enhanced in *jag nub* gynoecia (Fig. 6) (Ohno et al., 2004).

We next followed these defects throughout gynoecium development. In wild-type flowers, the gynoecium develops as a cylinder of tissue (Fig. 6A) (Bowman, 1993; Smyth et al., 1990). During this early stage, *jag nub* gynoecia develop notches near the apical end (Fig. 6B). As the gynoecium grows, these notches enlarge and appear to be restricted to the valve regions (Fig. 6C,D). At stage 11, the gynoecial cylinder closes with the formation of stigmatic papillae in the apical region (Fig. 6E). In *jag nub* mutants, however, the gynoecium does not close, leaving a gaping hole in the ovary (Fig. 6F). The valve tissue in this apical region is often shredded in appearance (Fig. 6H). These data show that *JAG* and *NUB* promote the growth of valve tissues during gynoecium development, and that failure of *jag nub* valves to enclose the ovules is caused by this defect. In agreement with this interpretation, we also have found that strongly affected *FIL::NUB* transgenic lines develop valve tissues that grow beyond their normal limits and expand into the style region, showing that *NUB* is also sufficient to ectopically activate valve growth (Fig. 8A,B). Interestingly, the epidermal cells of the ectopic valve tissue develop cuticular wax similar to epidermal cells found in the style region.

***NUB* promotes the proper differentiation of adaxial cell types in the valves**

The adaxial-specific expression of *NUB* during carpel development suggested that *NUB* may play a role in the development of tissues in this region. To explore this possibility, thin sections were made of wild-type, *jag*, *nub* and *jag nub* gynoecia (Fig. 7; data not shown). In wild type, the valves or carpel walls of the gynoecium are composed of six layers of cells: an outer epidermis, three layers of mesophyll, a modified layer of mesophyll termed the *enb* and finally the inner epidermal layer termed the *ena* (Fig. 7A). Most of the cell layers are established early during gynoecium development as the dome of tissue, which forms the gynoecium, begins to develop a cleft down the middle to create a cylinder of tissue at stage 7 (see Fig. S3 in the supplementary material) (Alvarez and Smyth, 1998). Later, during stage 9-11, cells near the valve margins of the valve divide, resulting in a final count of six cell layers. At flower opening (stage 13), each cell layer is distinguishable as a result of differences in cell size. The *enb* cells, for example, are very small owing to cell divisions that begin at stage 9. By contrast, cells of the *ena* are very large and also begin to differentiate in size from the other cell layers around stage 9.

In *jag nub* mutant gynoecia, we found that the number of cell layers was reduced (Fig. 7A,C). We measured cell layer number in two regions of the carpel walls and found a one to two

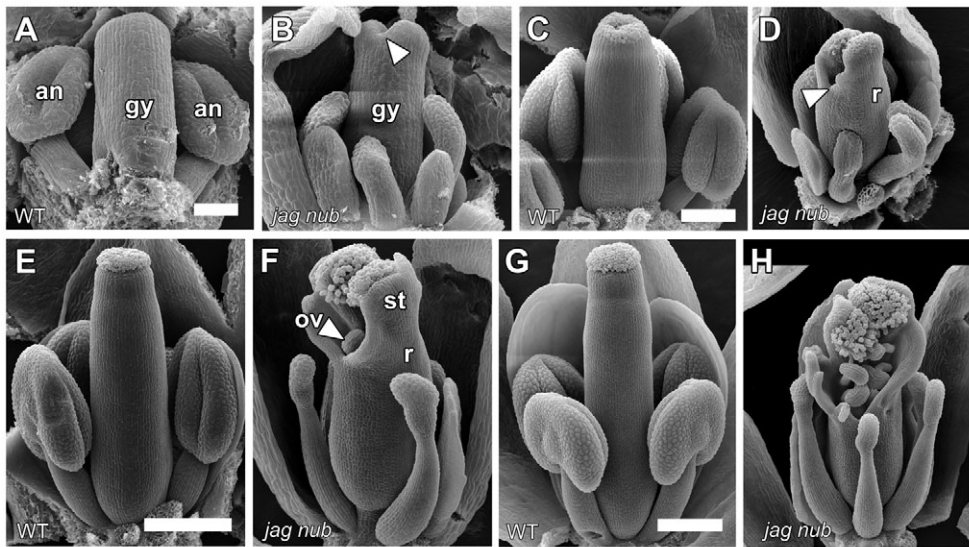


Fig. 6. Gynoecium development in wild type and *jag nub* mutants. (A-H) SEM images of gynoecia from stage 7 (A,B), stage 10 (C,D), stage 11 (E,F) and stage 12 (G,H) flowers from wild type (A,C,E,G) and *jag nub* mutants (B,D,F,H). Defects in valve growth in *jag nub* mutants are initially observed as small notches in the gynoecial cylinder (B, white arrowhead). Later, as the gynoecium grows apically, the notches widen and elongate, leading to the exposure of the normally internal ovules (D,F,H). an, anther; gy, gynoecium; r, replum; st, style. Scale bars: 50 μm for A,B; 100 μm for C,D; 200 μm for E-H.

cell layer reduction (Fig. 7D). This reduction always appeared to be at the expense of the mesophyll. These defects suggest that *JAG* and *NUB* are necessary to promote the cell divisions that established the correct number of cell layers. We also found that *jag* single mutants display a slight decrease in the number of cell layers, but not to the extent of *jag nub* mutants (Fig. 7A-D). We examined *nub* single mutants but did not observe any apparent defects in cell layer number or differentiation (data not shown)

The differentiation of specific cell types in the gynoecium is also affected in *jag nub* mutants. As mentioned before, cells in the *enb* layer undergo cell divisions during stage 9-12, resulting in cells with a very small width. Late in fruit development, these cells become lignified and contribute to fruit opening (Spence, 1996). In *jag nub* mutants, however, these cells are enlarged, presumably as a result of

the reduction in the number of cell divisions that occur (Fig. 7A,C,E). We examined *jag nub* fruit and could observe cells in the *enb* layer that were enlarged and did not stain for lignin (see Fig. S4 in the supplementary material). The overall size of the cells and the lack of lignification suggest that these cells may have acquired partial mesophyll identity. Only the largest cells of the *enb* layer tended to be unligified.

Development of the *ena* layer was also affected in *jag nub* mutants. Whereas *enb* layer cells were larger in *jag nub* mutants, *ena* cells were smaller than in wild type (Fig. 7A,C). Both the *ena* and *enb* defects are seen in *jag* single mutants, but to a lesser degree (Fig. 7A-C,E). The outer epidermal cells were slightly wider in *jag* and *jag nub* mutants compared to wild type (two-tailed *t*-test, $P=0.0025$ and 0.0210 , respectively), but there was no significant difference between *jag* and *jag nub* mutants ($P=0.8257$), consistent with the

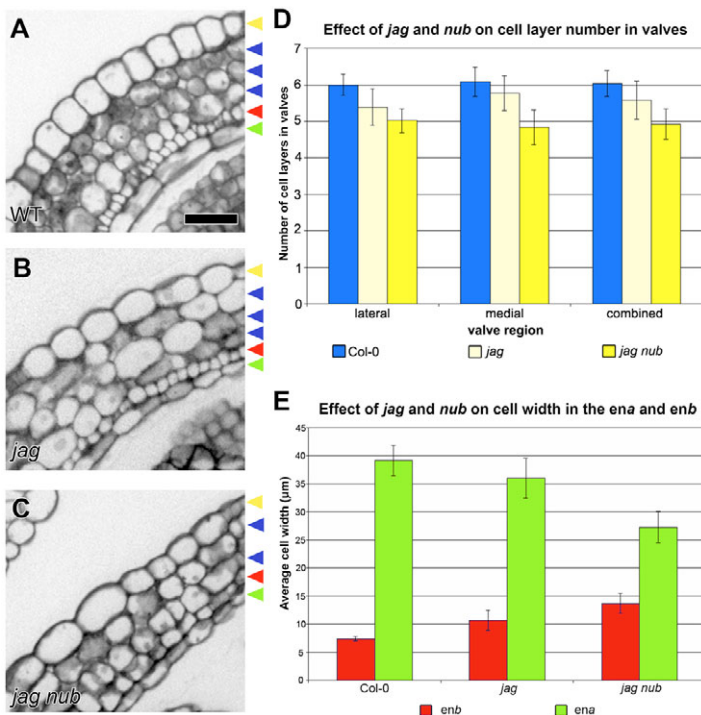


Fig. 7. Valve histogenesis is disrupted in *jag* and *jag nub* mutants. (A-C) Plastic-thin sections of valves from gynoecia of stage 13 flowers from wild type (A), *jag* (B) and *jag nub* (C) mutants. (A) Wild-type valves develop six tissue layers: an outer epidermal layer (yellow arrowhead), three layers of mesophyll (blue arrowheads), one layer of *enb* (red arrowhead) and a modified inner epidermal layer termed the *ena* (green arrowhead). (C) In *jag nub* mutants, one layer of mesophyll is often missing, and the *enb* and *ena* layers lack the characteristics that differentiate them from other tissue layers. (D) Graph showing the effects of the *jag* and *jag nub* mutations on cell-layer number in the medial and lateral valve regions. (E) Graph showing the effects of the *jag* and *jag nub* mutations on the average cell width in the *ena* and *enb* layers of the valves. Error bars indicate s.d. Scale bar: 25 μm.

lack of *NUB* expression in this cell layer (see Fig. S5 in the supplementary material). These data indicate that *JAG* and *NUB* play a specific role in promoting the differentiation of adaxial cell types in the carpel walls.

Ectopic *NUB* expression multiplies cell layer number in floral organs

JAG and *NUB* promote the cell divisions that establish the proper number of cell layers in the gynoecium. We explored the role of *JAG* and *NUB* in this process further by examining the effect of ectopic *NUB* expression on cell layer number in *FIL::NUB* transgenic lines. Consistent with our loss-of-function studies, we find that ectopic *NUB* expression is sufficient to promote the amplification of cell layer number in valves, sepals and petals. In carpels, most of the increase in cell layer number is restricted to tissues overlying the main vascular bundle (Fig. 8C,D). In sepals, the effect is more uneven (Fig. 8E,F). Petals, which normally have only two to three layers of mesophyll, develop up to six layers in

FIL::NUB (Fig. 8G,H). Although cell layer number is affected, no effect on the identity of the tissues that develop on the abaxial side can be seen in the flower. In the gynoecium, where *JAG* and *NUB* play a role in the differentiation of adaxial cell types, no ectopic *enb* or *ena* layer development is seen in *FIL::NUB* transgenic lines (Fig. 8C,D; data not shown). These data indicate that *JAG* and *NUB* may regulate the differentiation of adaxial-specific cell types in the gynoecium in parallel with genes that control tissue distribution, similar to their role in development of the microsporangia.

NUB promotes leaf blade growth redundantly with *JAG*

The development of ectopic blade tissue along the petioles of *jag-5D* rosette leaves demonstrated that *JAG* is sufficient to promote leaf blade growth. The blade area of wild-type leaves is, however, similar to that of *jag-1* mutants (Dinneny et al., 2004). More recently, we have shown that combining *jag-1* with the *fil* and *yabby3* (*yab3*) mutations results in a dramatic loss of blade development, demonstrating that *JAG* does indeed promote leaf blade expansion (Dinneny et al., 2005). When we examine the leaves of *jag nub* mutants, we also see a reduction in leaf blade area compared with wild type (Fig. 9A-D). The shape of *jag nub* leaves is somewhat variable and notches can form in the leaves (Fig. 9C). These notches may represent enhanced versions of the serrations found in *jag* single mutants (Fig. 9B). Leaves of *jag nub* mutants also develop outgrowths on the abaxial side (Fig. 9E-G). These outgrowths are often radialized and develop a high concentration of trichomes. In *kanadi* mutants, such outgrowths, termed enations, are also observed (Eshed et al., 2004). The enations of *jag nub* mutants are most common overlying vascular tissues, and tend to occur more frequently near the tips of the leaves and serrations. Scanning electron microscopy of the epidermal cells that constitute *jag nub* enations shows that they are shaped similarly to cells overlying vascular bundles (Fig. 9G).

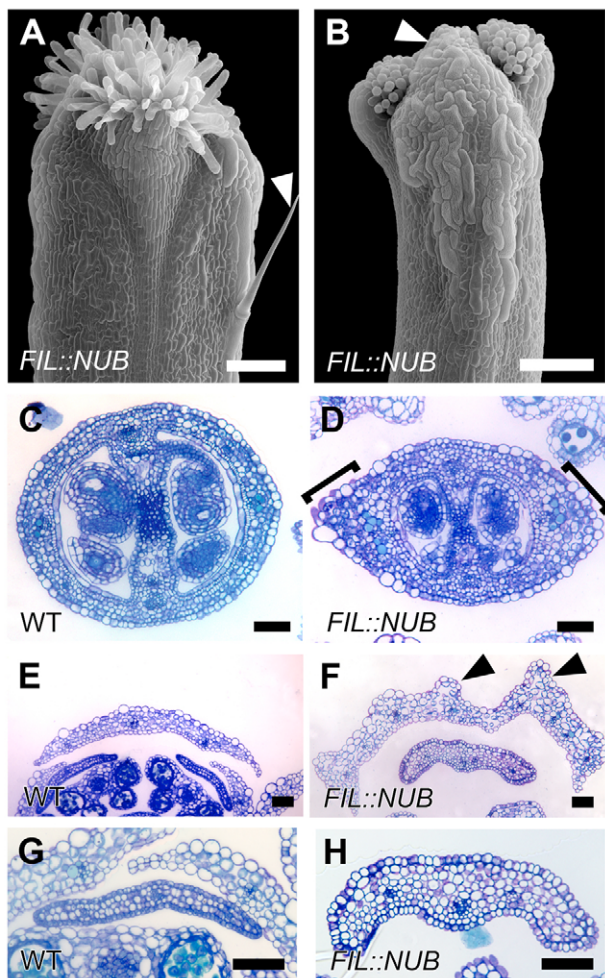


Fig. 8. Effects of the *FIL::NUB* transgene on valve growth and cell layer proliferation. (A,B) SEM images of stage 13 gynoecia from the JD148-A transgenic line taken from the replum side (A) and valve side (B). Note the extension of valve tissue into the style region (B, white arrowhead). (C-H) Plastic-thin sections of gynoecia (C,D), sepals (E,F) and petals (G,H) from wild type (C,E,G) and the *FIL::NUB* transgenic line JD148-A (D,F,H). Tissue layer proliferation is seen in the medial region of the valves (brackets), in irregular locations in sepals (arrowheads) and throughout the petals of JD148-A flowers. Scale bars: 50 μ m.

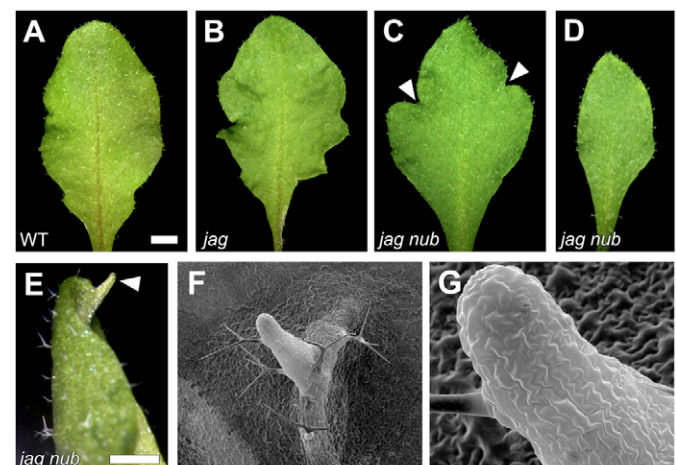


Fig. 9. Leaf phenotypes of *jag nub* mutants. (A-D) Fourth or fifth leaves from wild-type (A), *jag* (B) and *jag nub* (C, D) mutants. (C) Arrowheads point to notches in a *jag nub* leaf. (E) In *jag nub* mutants, leaves often develop radial outgrowth (enations, white arrowhead) on the abaxial side. These enations often develop near the tips of leaves and serrations. (F, G) SEM images of an enation emerging from the abaxial mid-vein of a *jag nub* leaf. Scale bars: 2 mm for A-D; 1 mm for E.

Abaxial *NUB* expression in leaves promotes trichome development

Although abaxial expression of *NUB* does not have an effect on the differentiation of abaxial cell types in the flower, we found that leaf epidermal development is affected. During early vegetative development in wild type, leaves form trichomes (leaf hairs) only on the adaxial surface (Fig. 10) (Kerstetter et al., 2001). After the plant has produced about six leaves, trichomes begin to develop on the abaxial side and increase in number until the formation of the cauline leaves. In both strong (Fig. 10) and moderate (see Fig. S6 in the supplementary material) *FIL::NUB* transgenic lines, trichome development is precocious in comparison with wild type. For example, the transgenic line JD148-A begins to develop abaxial trichomes as early as the third leaf. The early development of trichomes on the abaxial side of leaves suggests that the *FIL::NUB* transgene is able to impart partial adaxial identity to the abaxial side of leaves. We have also noticed that *FIL::NUB* transgenic lines occasionally develop trichomes on the adaxial side of floral organs, including petals, stamens and carpels (Fig. 8A; data not shown).

DISCUSSION

Early during lateral organ development in plants, a coordinate system is established that differentiates the adaxial side from the abaxial side. Soon afterwards and possibly coincident with organ polarization, organ outgrowth is initiated, marking the first morphogenetic event in the development of the lateral organ. In *Arabidopsis*, we have shown that the subsequent morphogenetic events that determine organ shape rely on the closely related *JAG* and *NUB* genes to specify organ domains competent for growth. In the absence of *JAG* and *NUB* activities, both morphogenesis and the subsequent late events of histogenesis fail to occur properly in lateral organs. Although *JAG* and *NUB* are able to activate morphogenesis and are necessary to promote the differentiation of cell types, they are not sufficient to promote the differentiation of these cell types in most cases. We therefore propose that *JAG* and *NUB* regulate cellular differentiation in cooperation with other factors that provide positional information.

JAG and *NUB* as shape determinants

In this work, we have extended our understanding of the role that *JAG* plays in shaping lateral organs by unraveling a layer of genetic redundancy caused by *NUB*, which masks the effects of *jag* mutations on stamen and carpel development. In stamens, *JAG* and *NUB* function together to promote microsporangia formation. Our analysis, using the expression of genes that mark different regions of the anther, reveals that the *jag nub* anther is composed largely of connective tissue. Interestingly, however, the initial expression pattern of the *SPL* microsporangia marker appears normal in *jag nub* anthers. These data suggest that *JAG* and *NUB* are not required to specify the identity of microsporangia tissues, but instead promote growth on the adaxial side, which is a prerequisite for microsporangia formation. In the gynoecium, *JAG* and *NUB* act to drive sufficient growth of the valves necessary to enclose the ovules. Growth defects in the gynoecium of *jag nub* mutants appear to be limited to the valves, while replum and style growth is unaffected. This function is the converse of that performed by genes such as *CRABS CLAW* and *SPATULA*, which affect the growth of stigma and style tissues without causing defects in valve growth (Alvarez and Smyth, 1999; Alvarez and Smyth, 2002).

Characterizing the redundant roles of *JAG* and *NUB* has allowed us to determine the relationship between their spatial patterns of gene expression and their activity. This is particularly important for

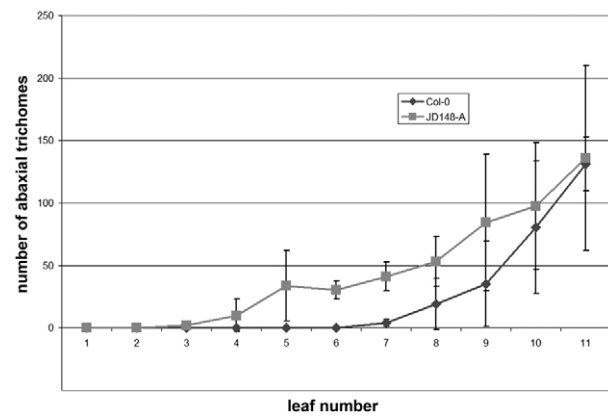


Fig. 10. The effect of abaxial *NUB* expression on the timing of trichome development in rosette leaves. Trichome number was counted on the abaxial side of leaves from plants that had just bolted. T2 segregating transgenic plants were scored. Four leaves were counted for each leaf class. Trichomes that developed overlying the main vascular bundles were not counted. Col-0, wild type; JD148-A, *FIL::NUB* transgenic line. Error bars indicate s.d.

determining directness, and to establish whether these genes control distinct morphogenetic events or act more globally to control growth throughout an organ. Although the expression of *JAG* suggests that it may play a role in stamen and carpel development, it was necessary to examine the function of *NUB* to elucidate the nature of this activity. Taking into account the mutant phenotypes of *jag* and *jag nub* mutants reveals that the expression domains of *JAG* and *NUB* directly correlate with defects in growth that occur when their functions are disrupted. Furthermore misexpression studies demonstrate that both *JAG* and *NUB* are able to activate growth in ectopic locations. Together, these data show that *JAG* and *NUB* control specific morphogenetic events during lateral organ development that are crucial for determining final shape.

The role of *JAG* and *NUB* in regulating adaxial development

Unlike *JAG*, which is expressed in all cell layers of lateral organs, *NUB* is limited to the adaxial side of lateral organs. Many studies have focused on elucidating the pathways that are involved in determining adaxial/abaxial polarity. Expressed on the abaxial side, the GARP-type transcription factors encoded by *KANADI 1*, *2* and *3* promote abaxial development by activating the expression of *FIL* and by negatively regulating the expression of adaxial promoting genes such as the miRNA-regulated class of HD-ZIP transcription factor genes, *PHB*, *PHAVOLUTA* and *REVOLUTA* (Emery et al., 2003; Eshed et al., 2001; Eshed et al., 2004; Kerstetter et al., 2001; Mallory et al., 2004; McConnell et al., 2001; Pekker et al., 2005). Ectopic expression of *PHB* has been shown to negatively regulate *FIL* expression, whereas ectopic *KAN* expression on the adaxial side can suppress *PHV* and *REV* expression. One interesting peculiarity with the expression domain of adaxial identity factors is that they are often expressed in both the meristem and the adaxial side of lateral organs. *NUB* expression, however, is limited to the adaxial side of lateral organs and is excluded from the meristem. This suggests that *NUB* may function downstream of, or in parallel to, the polarity pathways. Examination of *FIL* and *PHB* expression in *jag nub* mutants shows no clear mis-regulation of either gene in the gynoecium. In stamens, *FIL* expression appears to extend circumferentially into

the adaxial domain; however, this may simply be a consequence of the altered growth dynamics of a *jag nub* anther, where the normally proliferative adaxial microsporangia region is stunted. Furthermore, ectopic expression of *NUB* on the abaxial side does not suppress *FIL* expression, nor does it result in ectopic *PHB* expression (see Fig. S7 in the supplementary material). Consistent with these observations, phenotypic analysis of *NUB* gain-of-function and loss-of-function lines does not reveal any homeotic conversion of abaxial or adaxial tissues. These data suggest that *JAG* and *NUB* regulate histogenesis in parallel with pathways that establish polarity. The polarity pathways, which are known to regulate the spatial distribution of tissues, may require input from pathways that regulate growth. Although the nature of this relationship is unclear, recent work demonstrating that *FIL* and *YABBY3* act in cooperation with *JAG* to pattern the distribution of tissues in the fruit supports the existence of such a partnership (Dinneny et al., 2005). It may be that, in addition to promoting growth, *JAG* and *NUB* also promote the competence of tissues to respond to factors that determine tissue type and distribution, thus linking morphogenesis to histogenesis.

Two observations complicate any strict delineation of *JAG* and *NUB* function in adaxial development, however. First, *jag nub* mutants develop enations on the abaxial side of their leaves. Guided by the model proposed by Waites and Hudson, that the juxtaposition of adaxial and abaxial domains drives tissue outgrowth (Waites and Hudson, 1995), enation formation in *jag nub* leaves suggests a role for *JAG* and *NUB* in establishing/maintaining the boundaries between adaxial and abaxial halves. The second observation, that *NUB* expression on the abaxial side is sufficient to drive precocious trichome development further suggests that *NUB* may also play a more active role in regulating adaxial identity.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/9/1645/DC1>

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