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LAM-1 and *FAT* Genes Control Development of the Leaf Blade in *Nicotiana sylvestris*

Neil A. McHale

Department of Biochemistry and Genetics, The Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504

Leaf primordia of the *lam-1* mutant of *Nicotiana sylvestris* grow normally in length but remain bladeless throughout development. The blade initiation site is established at the normal time and position in *lam-1* primordia. Anticlinal divisions proceed normally in the outer L1 and L2 layers, but the inner L3 cells fail to establish the periclinal divisions that normally generate the middle mesophyll core. The *lam-1* mutation also blocks formation of blade mesophyll from distal L2 cells. This suggests that *LAM-1* controls a common step in initiation of blade tissue from the L2 and L3 lineage of the primordium. Another recessive mutation (*fat*) was isolated in *N. sylvestris* that induces abnormal periclinal divisions in the mesophyll during blade initiation and expansion. This generates a blade approximately twice its normal thickness by doubling the number of mesophyll cell layers from four to approximately eight. Presumably, the *fat* mutation defines a negative regulator involved in repression of periclinal divisions in the blade. The *lam-1 fat* double mutant shows radial proliferation of mesophyll cells at the blade initiation site. This produces a highly disorganized, club-shaped blade that appears to represent an additive effect of the *lam-1* and *fat* mutations on blade founder cells.

INTRODUCTION

Because dividing plant cells are fixed in their original positions by a rigid cell wall, morphogenesis hinges on strict controls over basic patterns of cell division (Gunning, 1982). In principle, this has at least two components: the first involves spatial control over the onset of meristematic activity and the second concerns the orientation of cell division. Initiation of leaf primordia by the shoot apical meristem is thought to involve coordinated changes in both aspects (Smith and Hake, 1992). Localized acceleration of cell division is observed 1.5 plastochrons (P) prior to emergence of the primordium in Pisum, but there is also a significant increase in the frequency of periclinal divisions at the site of an incipient primordium approximately half a plastochron before emergence (Lyndon, 1983).

Histological studies (Avery, 1933; Smith, 1934; Foster, 1936) show that initiation of the leaf blade along the lateral flanks of the primordium also involves spatially defined changes in the patterns of cell division. The blade mesophyll was originally thought to be derived from meristematic divisions in a single subepidermal file of initial cells by an alternating sequence of anticlinal and periclinal divisions (Avery, 1933). However, analysis of periclinal chimeras with genetically marked cells in the histogenic layers of the apical meristem revealed that all three layers of the primordium make a contribution to the blade (Stewart and Burk, 1970). Later analysis of clonal sectors induced by irradiation in the outer L2-derived layers of the leaf blade in tobacco demonstrated that the upper and lower mesophyll have a separate lineage emanating from at least six files of founder cells on the flank of the primordium (Poethig and Sussex, 1985b).

These studies have provided valuable insights into cellular parameters of leaf development; however, the molecular mechanisms remain largely unknown. Identification and cloning of genes governing essential steps in the leaf pathway should greatly accelerate progress toward this goal (Smith and Hake, 1992). Cloning and sequence analysis of the *Knotted-1* (*Kn1*) leaf mutation in maize (Hake et al., 1989; Vollbrecht et al., 1991) have provided the first example of a homeobox gene in plants. Patterns of *KN1* expression in mutant and wild-type plants suggest that this gene normally controls basic aspects of cell determination in the shoot apex during initiation of new primordia (Smith et al., 1992). Many other single mutations affecting leaf development have been described (Marx, 1983). Some produce subtle changes in size, form, and shape (Sinnott, 1960), but others affect major aspects of morphology, such as *lanceolate* in tomato (Caruso, 1968) and *unifoliata* in pea (Blixt, 1967) and bean (Roberts, 1982), which control formation of compound versus simple leaves. In addition, there are homeotic mutations producing full conversions of leaf type in pea, such as *afila* that converts leaflets to tendrils and *tl* that converts tendrils to leaflets (Meicenheimer et al., 1983). Histological analysis of the *afila* mutant indicates that this gene controls the activity of the adaxial and marginal meristems. In general, however, there has been little effort to relate leaf mutations to basic cellular mechanisms or to assign any temporal relationships in the leaf pathway.

I have initiated a genetic dissection of early events in formation of the leaf blade, with the primary focus on single genes controlling the cellular parameters of this pathway. Because green cells in the stem and midrib are photosynthetically competent, it was assumed that blade formation would be dispensable and, therefore, fully accessible to mutational analysis. The initial focus was on two major aspects of blade formation in which loss-of-function mutations were expected to produce definable cellular phenotypes: (1) specification of founder cells at the blade initiation site, and (2) patterns of cell division in the emerging blade. To assess the feasibility of this approach, a mutational analysis was conducted in diploid tobacco (*Nicotiana sylvestris*) in which cell lineage and basic patterns of division in the leaf are well established.

A recessive mutation was recovered (*lam-1*) that blocks formation of the leaf blade (McHale, 1992). Mutant leaves grow to their full adult length but never generate a leaf blade. Here, it is shown that the blade initiation site is established at the normal time and position in *lam-1* primordia. Anticlinal divisions proceed normally in the outer L1 and L2 layers at the flank of the primordium, but the inner L3 cells fail to establish the periclinal divisions that normally generate the middle mesophyll core. A second recessive mutation (*fat*) is described; it allows abnormal planes of cell division in all layers of the mesophyll during blade initiation and expansion. This mutation results in a leaf blade approximately twice its normal thickness due to addition of extra cell layers. The results suggest that *FAT* acts as a negative regulator with an essential role in the maintenance of defined cell layers in the expanding blade.

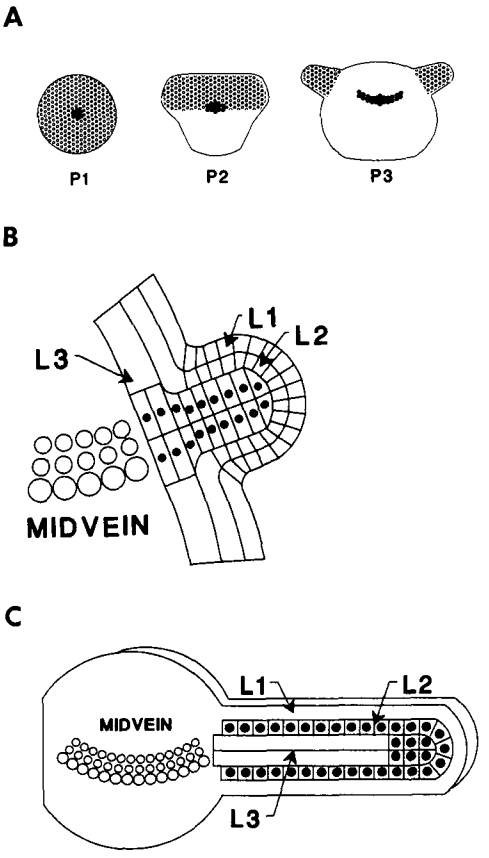

Figure 1. Blade Initiation and Expansion in Wild-Type *N. sylvestris* Leaves.

(A) Patterns of cell division and expansion in leaf primordia during the first three plastochrons (P1, P2, and P3).

(B) Patterns of cell division at the blade initiation site. The outer L1 and L2 cells divide in an anticlinal plane relative to the midrib. The inner L3 cells divide in a periclinal plane relative to the midrib.

(C) Cell lineage patterns in the expanded blade. The L2-derived cells (black circles) generate the upper and lower mesophyll in proximal regions of the blade and all four mesophyll layers in distal regions of the blade. L1 cells generate the upper and lower epidermis, and L3 cells generate proximal regions of middle mesophyll.

RESULTS

Blade Initiation

Tobacco leaf primordia pass through distinct phases of development marked by the establishment of regional identities, as shown in Figure 1A. Leaf age is given in plastochron (P) units, which define the time elapsed between initiation of successive primordia. During P1, the primordium is a conical projection, appearing circular in transverse view and showing uniform meristematic activity. The first evidence of dorsal/ventral identity is observed in P2. The adaxial side of the primordium maintains a rapid rate of cell division and flattens in a plane tangential to the shoot apical meristem. In contrast, cells on the abaxial side enter an expansion phase, generating a definable midrib. During P3, the adaxial side of the primordium is subdivided into blade and midrib domains. Cells on the adaxial surface above the midvein begin to expand, and isolated regions of meristematic cells are maintained at the lateral flanks that represent blade founder cells.

At its inception, the blade primordium consists of six superimposed cell layers generated by distinct division programs in the inner and outer cell layers, as shown in Figure 1B. The two internal cell layers of middle mesophyll are generated by a reorientation of cell division to the periclinal plane in L3 founder cells. Anticlinal divisions in the outer L1 and L2 cell layers form a sheath around the internal core of middle mesophyll. By wrapping around the L3 core, the outer L2 layer contributes two cell layers to the expanding blade, the upper (palisade) and lower (spongy) mesophyll. In a similar fashion, L1 produces the upper and lower epidermis (Dulieu, 1968; Stewart and Dermen, 1975; Poethig, 1984). Although proximal regions of the middle mesophyll are generated primarily by periclinal divisions in L3 cells, distal regions of the middle mesophyll can arise from periclinal divisions in L2 cells at the

leading edge of the blade (Stewart and Dermen, 1975), as shown in Figure 1C. This produces margin regions of varying size in which all internal layers of the blade are derived from the L2 lineage. In some cases, this pattern extends to the midrib, producing a blade mesophyll derived entirely from L2 cells.

lam-1 Mutant

The bladeless *lam-1* mutant in *N. sylvestris* is shown in Figure 2A (McHale, 1992). Mutant leaves are initiated in the usual helical pattern by the shoot apex and grow to their normal adult length without generating a blade. The scanning electron micrograph in Figure 2B shows that the morphology of emerging mutant primordia is normal. Mutant primordia at the P2 stage show a flattened adaxial surface, indicating normal dorsiventrality. Even though the failure in blade initiation occurs early in the development of mutant primordia (P3 stage, 0.5 mm), data in Figure 3 show that they grow in length at a rate similar to the wild type up to the 1-cm stage. Beyond this point, mutant leaves grow at a slower rate, but they attain a final length of 30 cm, indicating that programs governing longitudinal growth are unaffected by the mutation.

The transverse sections in Figures 4A and 4B show a wild-type and *lam-1* primordium at the P2 stage. The wild type and mutant show a flattened adaxial surface and bilateral symmetry typical of dorsiventral primordia. In addition, both show an onset of cell expansion on the abaxial side of the primordium, suggesting normal midrib development. The mutant primordium shows meristematic activity in the inner and outer cell layers at the lateral flanks (Figure 4B) but fails to generate the lateral projections observed at the flanks of the wild-type primordium (Figure 4A). Wild-type primordia at P3 show an onset of cell expansion on the adaxial side of the midvein and isolated groups of meristematic cells at the lateral flanks that represent the blade founder cells, as shown in Figure 4C. The emerging blade contains meristematic cells in the L1 and L2 layers that divide primarily in an anticlinal plane. The inner cell layers also contribute to the blade, dividing primarily in a periclinal plane and generating an inner core of middle mesophyll. The transverse section in Figure 4D shows that mutant primordia also establish a blade initiation site during P3. Cells on the adaxial side of the midvein show normal expansion and isolated groups of meristematic cells at the lateral flanks. Meristematic divisions at the initiation site, however, are largely confined to anticlinal divisions in the outer L1 and L2 layers. The inner L3 cells become vacuolated and appear to divide in random planes, ultimately failing to generate a middle mesophyll core. The mutant primordium still shows no evidence of lateral projections at the blade initiation site at this stage.

A small lateral projection is eventually produced at the initiation site in mutant primordia at the 1-cm stage, as shown in Figure 4E. This structure runs continuously from the tip to the base of the leaf in the normal position for the leaf blade but extends only 1 to 2 mm outward from the flank of the midrib. In later stages, it develops into a dark green strip of tissue, suggesting that it represents a vestigial blade. It appears that the vestigial blade in *lam-1* is generated primarily by periclinal divisions in distal L2 cells, although L3 cells may make some contribution as well. The longitudinal section through the vestigial blade in Figure 4F (6-cm leaf) suggests that the mesophyll cells maintain viability and continue dividing and expanding along with the epidermis as the leaf grows in length. Thus, in spite of the premature attenuation in lateral development

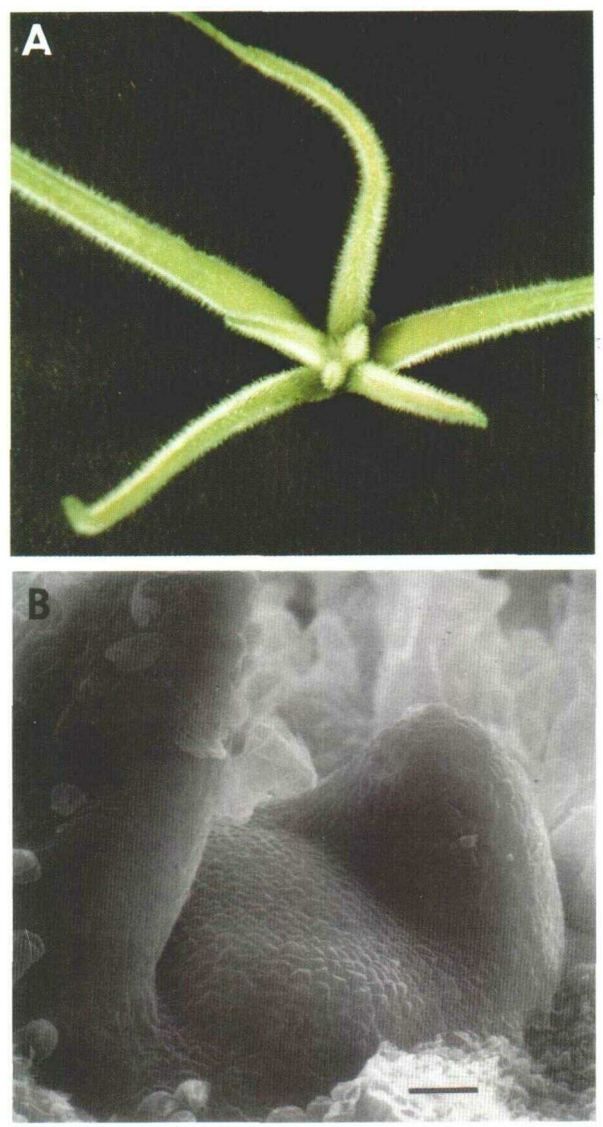

Figure 2. *lam-1* Mutant of *N. sylvestris* Showing Morphology of Emerging Primordia.

(A) Adult vegetative shoot of the *lam-1* mutant.
(B) Shoot apical meristem of the *lam-1* mutant. Bar = 30 μm.

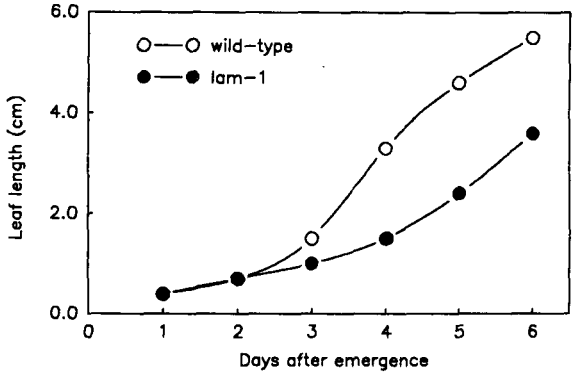

Figure 3. Leaf Growth in the Wild Type and *lam-1* Mutant of *N. sylvestris*.

Data represent the average length of the fourth true leaf on 12 seedling plants grown under continuous illumination.

of the blade, it appears that the mesophyll cells are competent to sustain longitudinal division and expansion.

fat Mutant

Another recessive mutation affecting cell division in the leaf blade of *N. sylvestris* was recovered by ethylmethane sulfonate (EMS) mutagenesis. This mutation was designated *fat*, because it was initially recognized as producing thick, dark green leaves that fail to attain full expansion, as shown in Figure 5B. A matched wild-type leaf is shown in Figure 5A for comparison. The mutant leaf grows normally in length and its width is similar to the wild type at the tip and base. The mutant leaf remains narrow (7 to 8 cm) in the middle region; however, this is where blade expansion is normally most pronounced in the wild type (12 to 15 cm). Measurements of leaf growth indicate that *fat* leaves grow normally in length and width up to the 2-cm stage, as shown in Table 1. Beyond this point, they show a deficiency in lateral development relative to the wild type. Mutant leaves grow to full adult length but attain just over half the width of the wild type.

Six cell layers are established during blade initiation in the wild type, as shown in the transverse section in Figure 5C. These six layers are maintained during subsequent expansion by anticlinal divisions relative to the blade surface. The transverse section through a *fat* primordium in Figure 5D shows that the mutation generates extra cell layers at an early stage in blade initiation. The emerging *fat* blade contains up to four layers of middle mesophyll, which appear to result from abnormal periclinal divisions in L2 or L3 founder cells.

During blade expansion, abnormal periclinal divisions are observed in all layers of the mesophyll, as shown in Figure 5E. Periclinal divisions typically involve clusters of three to six cells. Displaced clusters resume anticlinal divisions in their new location, becoming intercalated between existing layers. This process generates a blade approximately twice its normal thickness by doubling the number of mesophyll cell layers from four to approximately eight. In contrast to proximal regions in which mesophyll cell layers are maintained, distal regions of the *fat* blade are highly disorganized, as shown in Figure 5F. Mesophyll cells appear to divide in random planes, generating a blade margin with up to 15 cells in the dorsal/ventral axis. This region shows no evidence of defined cell layers.

lam-1 fat Double Mutant

To examine the combined effects of these mutations, appropriate crosses were conducted to generate the *lam-1 fat* double mutant. Emerging leaves of the double mutant have a bladeless phenotype and initially show a pattern of cell division that is indistinguishable from *lam-1*. Double mutant primordia show a normal onset of meristematic activity in the inner and outer cell layers at the blade initiation site in the P2 stage, as shown in Figure 6A. Double mutant primordia at P3 also display normal development of the midrib, as shown in Figure 6B. Cell expansion is observed on the adaxial and abaxial side of the midvein, and isolated groups of meristematic cells are observed at the lateral flanks. Unlike *lam-1* in which the inner cell layers become vacuolated, double mutant primordia show broad regions of meristematic activity in this region. The cells divide in random planes, however, and the primordium fails to generate a lateral projection in this phase. Meristematic activity declines by the 1-cm stage, and the cells undergo considerable vacuolation and lateral expansion, as shown in Figure 6C. This region develops into a broad lateral projection at the flank of the primordium. Continued cell division and expansion sustain radial proliferation of the presumptive mesophyll cells in this structure, as shown in the double mutant primordium at the 2-cm stage in Figure 6D. Cell number increases in a radial pattern outward from the midrib, generating a vestigial blade with up to 20 cells in the lateral axis and up to 30 cells in the dorsal/ventral axis. The vestigial blade shows no evidence of defined cell layers and has a highly disorganized network of secondary veins.

DISCUSSION

Blade initiation begins with the selection of a group of founder cells at the junction of the adaxial and abaxial surface of the primordium (Poethig and Sussex, 1985a). This is followed by a second phase in which a lateral projection is generated by a combination of anticlinal divisions in the outer L1 and L2 cell layers and periclinal divisions in the inner L3-derived layers. Anatomical observations suggest that the initial phase involving selection of founder cells proceeds normally in the *lam-1* mutant. Mutant primordia show a normal appearance of meristematic cells at the initiation site (Figures 4B and 4D). The first evidence of a defect is the failure of periclinal divisions

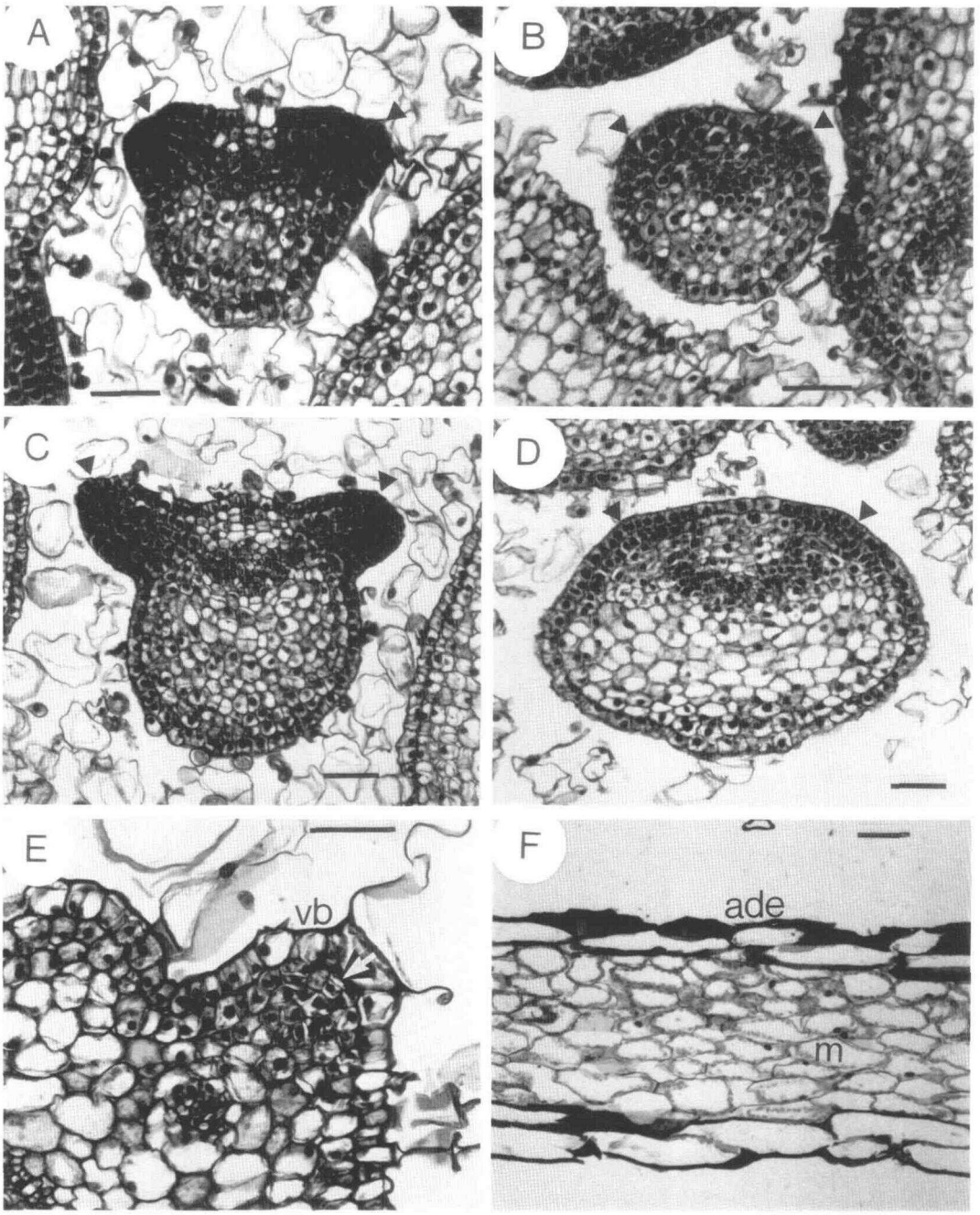

Figure 4. Blade Initiation in Wild Type and the *lam-1* Mutant of *N. sylvestris*.

(A) and **(B)** Anatomy of the wild-type and *lam-1* mutant leaves, respectively, at the P2 stage. Arrowheads show the blade initiation sites at the lateral flanks of the primordia. Bars = 50 μm.

(C) and **(D)** Anatomy of the wild-type and *lam-1* mutant leaves, respectively, at the P3 stage. Arrowheads show the blade initiation sites. Bars = 50 μm.

(E) Vestigial blade initiation in a *lam-1* primordium at the 1-cm stage. Arrow shows periclinal divisions in L2 cells at the blade initiation site. Bar = 50 μm. vb, vestigial blade.

(F) Anatomy of the vestigial *lam-1* blade at the 6-cm stage. Bar = 50 μm. ade, adaxial epidermis; m, mesophyll.

In **(A)** to **(E)**, the light micrographs represent median transverse sections of the primordium. The samples were taken from the shoot apex of wild-type and *lam-1* mutant plants in the juvenile, vegetative phase of development. The micrograph in **(F)** represents a median longitudinal section through the leading edge of the vestigial *lam-1* blade.

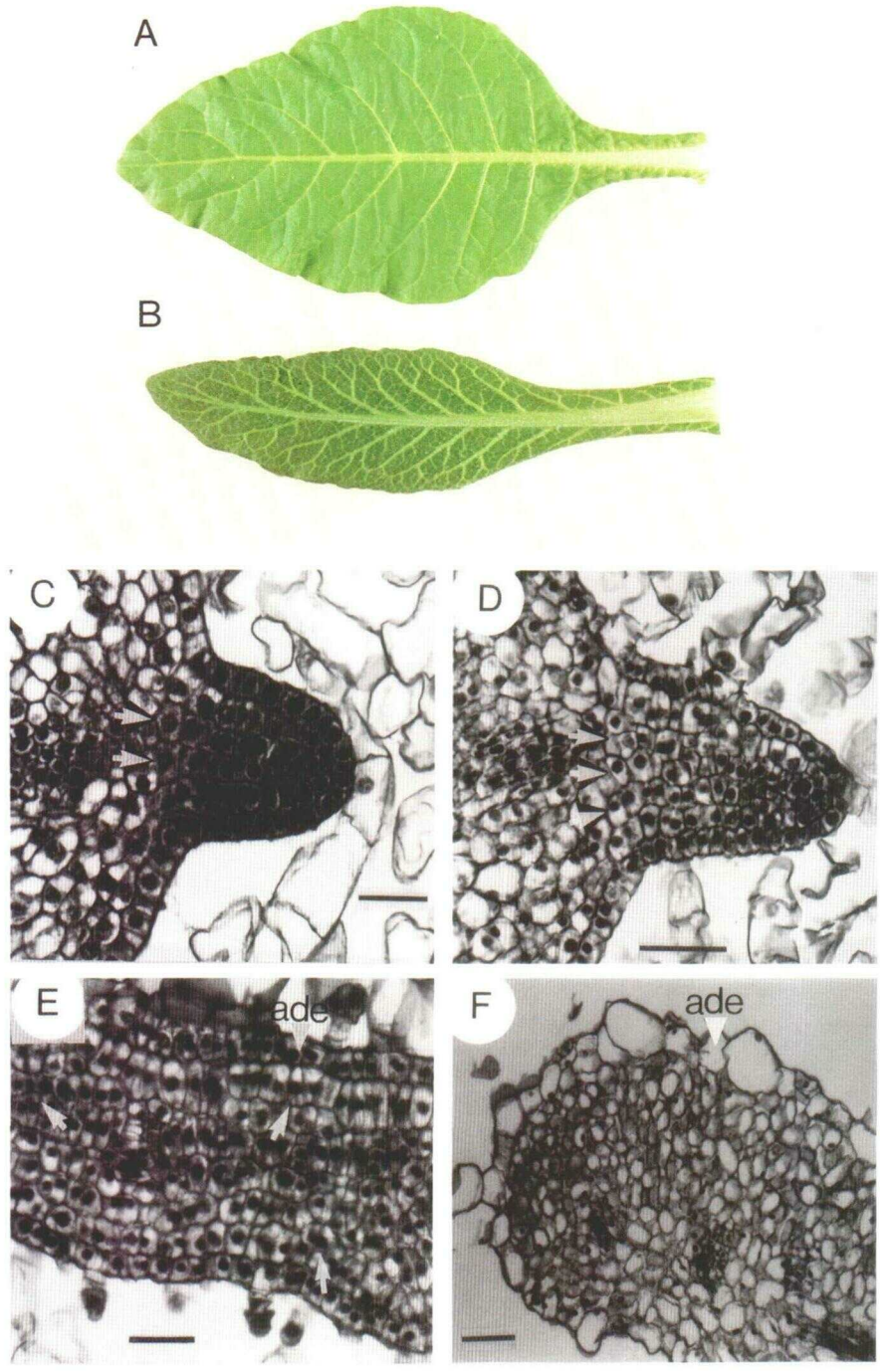

Figure 5. Leaf Morphology and Blade Anatomy in Wild Type and the *fat* Mutant of *N. sylvestris.*

(A) Wild-type leaf.
(B) *fat* mutant leaf.
(C) Blade initiation in a wild-type primordium at P3. Arrows show two files of middle mesophyll at the blade initiation site.
(D) Blade initiation in a *fat* mutant primordium at P3. Arrows show three files of middle mesophyll at the blade initiation site.
(E) Abnormal periclinal divisions in the blade mesophyll of a *fat* mutant leaf at the 1-cm stage. Arrows show periclinal cell division in the upper and middle mesophyll layers.
(F) Abnormal proliferation of mesophyll cells at the margin of a *fat* mutant leaf at the 4-cm stage.
In **(C)** to **(F)**, the light micrographs represent median transverse sections of the primordium. The samples were taken from the shoot apex of wild-type and *fat* mutant plants in the juvenile, vegetative phase of development. Bars = 50 μm. ade, adaxial epidermis.

Table 1. Leaf Growth in the *fat* Mutant and the Wild-Type Control

	Leaf Size (cm)[a]			
	fat		Wild Type	
Day	L	W	L	W
2	1.9	0.5	1.8	0.6
3	3.2	0.9	3.0	1.2
4	4.0	1.3	4.3	1.8
5	5.9	1.7	5.8	2.4
6	8.4	2.3	8.4	3.5
7	9.8	3.3	10.1	5.8
maturity	28.3	8.8	27.1	14.3

[a] Daily measurements of leaf length (L) and width (W) were taken following the emergence of leaves from the apex in eight plants for each genotype.

in the inner L3 cells during the P3 stage. The L3 cells show random planes of division, in contrast to the wild type in which periclinal divisions generate two distinct files of middle mesophyll (Figures 1B and 4C). Anticlinal divisions in the outer L1 and L2 layers of *lam-1* proceed normally, suggesting that the mutation has its primary effect in the inner cell layers during this phase. The *lam-1* mutation may interfere with the acquisition of middle mesophyll identity, blocking subsequent steps in the development of this region. Alternatively, it is possible that this gene has a direct role in the reorientation of cell division to the periclinal plane in founder cells of the middle mesophyll.

The defect in formation of the middle mesophyll at the P3 stage in *lam-1* primordia is correlated with a failure in emergence of a lateral projection at the blade initiation site (Figure 4D). This raises the possibility that formation of a middle mesophyll core by L3-derived cells plays a fundamental role

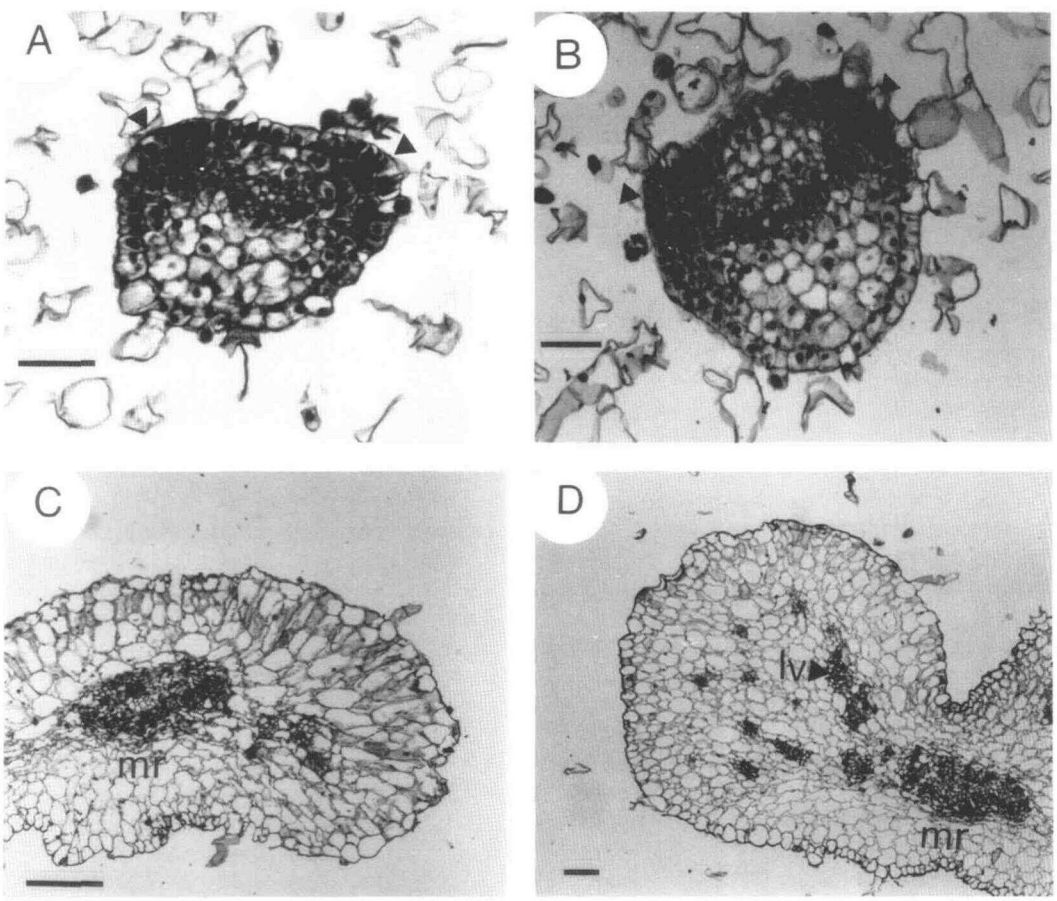

Figure 6. Blade Formation in the *lam-1 fat* Double Mutant of *N. sylvestris*.

(A) and **(B)** The P2 and P3 stages. Arrowheads show the blade initiation sites at the lateral flanks of the primordia. Bars = 50 μm.
(C) The 1-cm stage. Bar = 50 μm. mr, midrib.
(D) The 2-cm stage. Bar = 100 μm. mr, midrib; lv, lateral vein.
The light micrographs represent median transverse sections of the primordium. The samples were taken from the shoot apex of a *lam-1 fat* double mutant in the juvenile vegetative phase of development.

in blade initiation. The idea that internal cells play a controlling role in early stages of primordium development is supported by a recent analysis of periclinal chimeras in tomato (Szymkowiak and Sussex, 1992). The presence of the *fasciated* mutation in the L3 layer induces initiation of extra carpel primordia in the floral meristem. Similarly, it has been shown that the presence of the *KN1* mutation in cells of the middle mesophyll can induce surface cells to generate abnormal knot-like projections along the lateral veins of maize leaves (Sinha and Hake, 1990). Blade initiation in dicots may follow a similar pattern in which emergence of a lateral projection from the midrib is controlled by fundamental events in the inner cell layers. In this view, the L3-derived region at the flank of the primordium may represent a domain for expression of genes governing the early events in blade initiation.

Proximal regions of middle mesophyll are generated by periclinal divisions in L3 cells, but distal regions of middle mesophyll can arise from periclinal divisions in L2 cells at the leading edge of the blade (Stewart and Dermen, 1975). Together with anticlinal divisions in L2 cells, this process can generate large regions of blade mesophyll that are derived entirely from the L2 lineage. That *lam-1* leaves remain bladeless throughout development indicates that the mutation blocks development of blade tissue from the L2 as well as the L3 lineage. This suggests that *LAM-1* controls a common step in these two pathways. Expression of the *LAM-1* gene may be an essential step in formation of middle mesophyll tissue from either lineage. A series of periclinal chimeras carrying the *lam-1* mutation in individual layers of the apical meristem is being generated to address questions concerning the layer-specific roles of this gene in blade formation.

The molecular mechanisms controlling the orientation of cell division in plants are unknown. Genetic analysis of cell division in *Escherichia coli* shows that localization of the division site is controlled by a specific set of genes (Wang et al., 1991). Activation of the division process itself requires transcription of the temperature-sensitive filamentation (*fts*) Q, A, Z gene cluster, but localization of the division site is controlled by the negative regulators minicell C and D, which confer global inhibition of septation except at the midcell. Analysis of the *fat* mutation in *N. sylvestris* indicates that negative regulators may also play a role in plant cell division. Cell layers of the leaf blade are established during initiation and maintained as single cell layers by a strict pattern of anticlinal divisions relative to the blade surface (Figure 1). In the *fat* mutant, abnormal periclinal divisions are observed in all layers of the blade mesophyll during initiation and subsequent expansion (Figure 6). Because the *fat* mutation is recessive, it presumably defines a gene involved in repression of periclinal divisions in the blade. Plant cells establish the position of new cell walls prior to the onset of mitosis through a mechanism involving alignment of microtubules in the cell cortex (Gunning, 1982). It is possible that *FAT* normally acts as a negative regulator of this process.

Leaves of the *fat* mutant grow normally in length throughout development, but the blade shows a significant deficiency in lateral expansion starting when the leaf is ~2 cm in length (Table 1). At maturity, the mutant blade is just over half the width of the wild type. Restricted lateral expansion of the mutant blade may be a consequence of periclinal divisions in the mesophyll and the subsequent intercalation of cells between existing layers. Displaced cells may encounter physical constraints in lateral division or expansion that restrict the normal growth of the blade. Alternatively, it is possible that cell displacement disrupts the normal patterns of cell–cell communication in the expanding mesophyll.

Radial proliferation of the blade mesophyll in the *lam-1 fat* double mutant appears to represent an additive effect of these mutations. Double mutant primordia show broad regions of meristematic activity in the internal cell layers at the initiation site (Figure 6B); this is in contrast to *lam-1* primordia in which internal cells are highly vacuolated at this stage (Figure 4D). This response is most likely a consequence of the *fat* mutation. Abnormal proliferation of mesophyll cells at the initiation site was observed in the *fat* mutant, primarily in the form of extra files of middle mesophyll (Figure 5B). In the double mutant, it appears that these presumptive mesophyll cells divide in random planes, failing to generate a defined middle mesophyll core. Because *fat* alone does not interfere with formation of a middle mesophyll core (Figure 5B), this phenotype in the double mutant presumably stems from the *lam-1* mutation. In summary, the current working model is that *fat* induces abnormal proliferation of mesophyll cells at the blade initiation site, but *lam-1* precludes the organization of these cells into a defined middle mesophyll core.

Various lines of morphological, anatomical, and genetic evidence suggest that flowers are essentially modified leaves (Steeves and Sussex, 1989). Recent work with floral identity genes in Arabidopsis and Antirrhinum strongly supports the idea that the leaf pathway represents a developmental ground state (Coen and Meyerowitz, 1991). Mutational dissection of leaf development will be an important step in defining fundamental aspects of organogenesis in plants. Unfortunately, mutations blocking early steps in leaf initiation may be lethal, precluding a straightforward genetic approach (Freeling, 1992). Blade initiation shows striking similarities to leaf initiation at the cellular level. Both structures are generated by a localized onset of periclinal divisions in inner layers and anticlinal divisions in surface layers. Because the blade is dispensable, it should be possible to dissect the earliest events in the formation of this lateral appendage. This should provide important insights into plant morphogenesis and may open avenues to the isolation of functionally related genes controlling leaf initiation in the shoot apical meristem.

METHODS

Mutant Isolation

The mutants were isolated in the M_2 generation following seed mutagenesis with ethylmethane sulfonate (EMS). Seeds of *Nicotiana*

sylvestris were soaked in water for 12 hr and exposed to 0.4% EMS for 2 hr. Plants were grown to maturity in the field for collection of M_2 seeds (McHale et al., 1988). The *fat* mutant was initially recognized as a variant with dark green cotyledons segregating at a frequency of ~10% in its M_2 family. Mutants were raised to maturity and backcrossed to the wild type, producing F_1 progeny with a normal phenotype. Self-pollination of several F_1 plants produced a pooled F_2 progeny that segregated 595 wild type to 140 *fat* mutants (~4:1), indicating a deviation from the expected 3:1 ratio for a single recessive mutation. Because the observed ratio is far removed from the 15:1 ratio expected for two recessive mutations, it was assumed that *fat* is a single mutation. The homozygous *fat* genotype is presumably underrepresented in the F_2 progenies due to occasional lethality.

lam-1 fat Double Mutant

The double mutant was generated by crossing mutant plants to produce a double heterozygote, which was raised to flowering and self-pollinated. Putative double mutants were identified in the F_2 progeny as rare variants in which the leaves initially appeared bladeless, but later showed an apparent overproduction of the vestigial blade tissue normally observed in *lam-1* mutants. To confirm the genotype, F_2 siblings showing the *fat* phenotype were grown for self-pollination to identify *fat/fat LAM-1/lam-1* individuals based on segregation of bladeless variants. One of the F_3 progenies was segregating *fat* and bladeless (double mutant) individuals that showed overproduction of the vestigial blade. The *fat* and double mutant classes appeared in a frequency of 171 to 96 (~2:1), indicating a deviation from the expected 3:1 ratio. As observed in previous crosses with *fat*, this shows a shortage of *fat* homozygotes, which presumably reflects occasional lethality for this genotype.

Histology

Plant materials were prepared for sectioning by overnight fixation in ethanol/chloroform/glacial acetic acid (6:3:1 v/v), followed by stepwise infiltration first with butanol and then with melted paraffin at 60°C in a vacuum oven. Sections (8 μm) were cut from paraffin blocks, fixed to glass slides, and dried overnight prior to staining with hemalum and safranin (Berlyn and Miksche, 1976).

Scanning Electron Microscopy

Samples were prepared for scanning electron microscopy by overnight fixation in ethanol/chloroform/glacial acetic acid (6:3:1 v/v) that was followed by stepwise transfer to 100% ethanol and critical point drying with liquid carbon dioxide in a Polaron pressure chamber (Watford, UK). Dried samples were mounted on stubs, sputter coated in an SPI Sputter (Westchester, PA), and examined with a scanning electron microscope (ISI-SS40; Topcon, Pleasanton, CA).

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