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Genetic analysis of leaf development in cotton

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Summary

Leaf shape in cotton is regulated by the developmental age of the shoot and by several major genes that affect leaf lobing. The effect of these factors was investigated by allometric analysis, cell lineage analysis, and by studying the expression of the leaf shape mutation, *Okra*, in genetic mosaics. Allometric analysis of leaf growth suggests that leaf shape is determined during the initiation of the primordium rather than during the expansion phase of leaf growth. Clonal analysis demonstrates that both the rate and duration of cell division are fairly uniform throughout the leaf. Cells in the marginal region of the developing cotton leaf contribute more to the growth of the lamina than they do in tobacco. The *Okra* mutation acts early in the development of a leaf and appears to accentuate a developmental pattern that is also responsible for heteroblastic variation in leaf shape. The expression of this mutation in genetic mosaics demonstrates that its effect does not diffuse laterally within the leaf primordium.

Key words: cotton, leaf shape, allometric analysis, clonal analysis, *okra* mutation, genetic mosaics.

Introduction

Leaves are generally planar, morphologically simple, determinate organs that are produced at the surface of shoot meristems throughout the development of a plant. Variation in leaf shape occurs both within and between species and is a typical feature of shoot development (Ashby, 1948; Allsopp, 1967). In addition to this genetically and developmentally regulated variation in shape, leaf shape is significantly affected by diverse environmental factors such as defoliation, photoperiod, light quality, and water potential (Ashby, 1950; Njoku, 1956*a*,*b*, 1957). The regulation of leaf morphogenesis therefore impinges upon many aspects of plant biology and for this reason represents a fascinating and complex problem in pattern formation in plants.

We are interested in determining the cellular basis of leaf morphogenesis. Initial studies focused on leaf development in tobacco (*Nicotiana tabacum* L.) since this species has been historically used as a model system for leaf development (Avery, 1933). More recently we have begun to explore the mechanism of leaf development in cotton (*Gossypium barbadense* L.), in part to determine the generality of the developmental parameters observed in tobacco, and also because cotton has several important advantages as an experimental system. Cotton grows rapidly, has morphologically simple leaves, and exhibits pronounced heteroblasty (serial change in leaf shape along the shoot). Mutations specifically affecting leaf shape have been identified and their effects on the growth pattern of the leaf and on the pattern of heteroblastic development have been characterized (Hammond, 1941*a*,*b*; Stephens, 1944*a*,*b*,*c*). In addition, mutations exist that are useful for clonal analysis of cell lineage, including a genetic trait (*Semigamy*) that produces individuals that are genetic mosaics for paternally derived and maternally derived cell lineages (Turcotte and Feaster, 1963; Turcotte and Feaster, 1967; Endrizzi *et al.* 1984; Stelly and Rooney, 1989). In this paper we will summarize what is known about the mechanism of leaf morphogenesis in cotton, and compare and contrast this with leaf morphogenesis in tobacco.

The cell lineage of the leaf

Lineage information obtained from periclinal chimeras of a number of dicotyledonous species indicates that leaves are derived from the three germ layers of the apical meristem (Satina *et al.* 1940; Satina and Blakeslee, 1941; Dulieu, 1968; Stewart and Burk, 1970; Stewart and Dermen, 1975). The L1 layer of the meristem gives rise solely to the epidermis of the leaf, the L2 (subepidermal) layer gives rise to the upper palisade and the lower spongy mesophyll near the center of the leaf and all of the mesophyll at the leaf margin. The L3 layer of the meristem produces the middle mesophyll layers in the central portion of the leaf (Stewart and Dermen, 1975). It should be noted that these chimeras do not demonstrate that the leaf primordium is derived from only three layers of cells in the shoot meristem because the L3 lineage encompasses several cell layers in the meristem, while the L1 and L2 each constitute a single cell layer.

Clonal analysis in tobacco has revealed that the leaf axis arises from about 100 cells in three layers of the primordium (Poethig and Sussex, 1985*a*,*b*). The lamina (expanded part of the leaf) develops from several rows of cells in three cell layers along the margin of the axis. In dicotyledonous leaves the cells of the outermost layer (L1 or dermatogen) give rise exclusively to the single cell layered epidermis by dividing anticlinally (Esau, 1965). This may be deduced from the orientation of cell plates in this region early in development and also from chimeras, although very little quantitative information exists on the nature of these divisions (Maksymowych and Erickson, 1960; Fuchs, 1968). It is interesting to note that the epidermis of the scale leaves of a number of dicotyledonous species undergoes periclinal divisions (Foster, 1936). In these cases the protodermis contributes to the mesophyll tissue, a phenomenon that does not occur in the lamina of more complex leaves.

The lineage of the L1 in the leaves of monocotyledons exhibits a great deal of variation. Histological studies and genetic mosaics have shown that the outermost cell layer of the developing leaf in a variety of monocotyledonous taxa undergoes periclinal divisions early in development (Sharman, 1942, 1945; Mericle, 1950; Pray, 1957; Stewart and Dermen, 1979; Poethig, 1984). In contrast, genetic mosaics of broad leaf monocots such as banana (*Musa paradisiaca* L. cv. Vittata) indicate that cells derived from the epidermis do not contribute to the growth of the lamina (Stewart and Dermen, 1979).

It has been suggested that the internal cells of the dicotyledonous leaf are derived from a small group of marginal cells known as the marginal initials (Avery, 1933). Avery (1933) concluded that a single marginal cell gives rise to both the upper and lower mesophyll of the tobacco leaf and is the ultimate source of all the internal tissue of the leaf. These conclusions were based upon histological studies of early leaf development and were devoid of either lineage information or quantitative measurements of rates or orientation of cell division. A marginal meristem model of lamina development makes several predictions: (1) cell divisions would be more frequent near the margin than in an internal region; (2) a clonal sector produced at the leaf margin early in development would extend from the margin inward; (3) a sector induced early in development will extend further into the lamina than a sector produced later in development.

These predictions have been tested and, in general, are not supported by existing data. Evidence against the model was provided initially by Maksymowych and Erickson (1960). This study showed that the orientation of cell division near the leaf margin in *Xanthium italicum* Moretti was not compatible with the predicted meristematic function of this region of the leaf. Subsequent studies of mitotic frequencies during leaf development in tobacco (Dubuc-Lebreux and Sattler, 1980) indicated that the rate of cell division is elevated a distance from the margin (the plate meristem region of the lamina) rather than in the marginal region. Clonal analysis of lamina expansion in tobacco also failed to corroborate the model since marginal sectors were not oriented as the model would predict (Poethig and Sussex, 1985*b*). Sectors located at the leaf margin were oriented parallel to the margin rather than perpendicular to it, implying that cell division in this region serves primarily to accommodate the growth of the internal tissue of the leaf and does not contribute to the expansion of the lamina. Their analysis also suggested that the rate of cell division is elevated in interveinal regions of the lamina, rather than than in a narrow marginal region.

Clonal analysis of cell division patterns in developing cotton leaves lends support to the hypothesis that the leaf margin does not contribute disproportionately to the growth of the lamina. Nonetheless, the orientation of cell division at the leaf margin in cotton is significantly different from that of tobacco. This study was conducted by γ-irradiating the third leaf of *G. barbadense* L. (American Pima Cotton) plants homozygous for the *virescent7* (v_7) mutation at various stages of leaf development. Because *G. barbadense* L. is an allotetraploid species, the effect of this chorophyll mutation depends on the ratio of mutant and wild-type alleles at this locus and at the corresponding locus in the homeologous genome (Turcotte and Feaster, 1973). Chromosomal rearrangements that result in the loss of a mutant v_7 allele or a wild-type homeologous allele produce, respectively, green and white sectors in mature leaves (Barrow *et al.* 1973; Barrow and Dunford, 1974). A representative leaf from these irradiations is shown in Fig. 1.

The orientation of cell division at the leaf margin is perhaps the most striking feature of the cell division pattern during leaf morphogenesis in cotton. Mesophyll sectors originating at the margin of the leaf tend to be oriented perpendicular to the margin, rather than parallel to the margin as is the case in tobacco (Fig. 2). In this respect, therefore, the cell division pattern at the margin of a cotton leaf is consistent with the classical

Fig. 1. γ-ray induced sectors on a third leaf, irradiated after initiation of the lamina

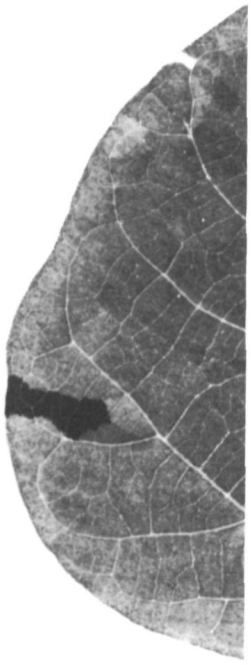

Fig. 2. Marginal sectors induced early in lamina development on the sixth leaf of a cotton plant. Note that the orientation of the long axis of the two marginal sectors is perpendicular to the margin.

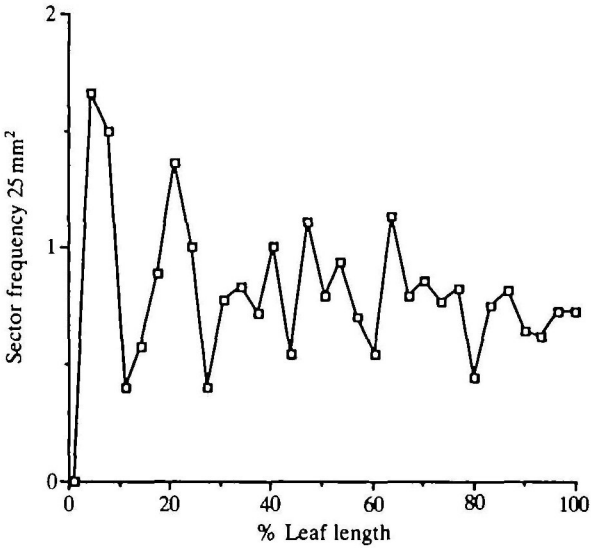

Fig. 3. The average sector frequency (sectors/25 mm^2) along a leaf irradiated at approximately 1 mm in length. At this stage the lamina had already been initiated.

model of leaf development in which the cells of the lamina are ultimately derived from marginal cells (Avery, 1933). However, the fact that marginal sectors do not appear to be larger than sectors elsewhere in the lamina (data not shown) indicates that cell divisions in the marginal region of a cotton leaf do not contribute disproportionately to the expansion of the lamina. Furthermore, if marginal initials function as Avery suggested then marginal sectors should extend into both the upper and lower mesophyll. This class of sectors is conspicuously absent in both cotton and tobacco, indicating that the upper mesophyll and lower mesophyll layers are derived from different populations of cells in the leaf axis.

The distribution of sectors produced in a cotton leaf that was irradiated at a length of approximately 1 mm is shown in Figs 3 and 4. Assuming that sector frequency is proportional to the frequency of cell division, as has been shown to be true in tobacco (Poethig and Sussex, 1985*b*), these data reveal that the frequency of cell division is relatively uniform throughout the lamina. With the exception of the tip, which has a relatively high sector frequency, the average number of sectors per unit area decreases only slightly from tip to base of the cotton leaf (Fig. 3). In tobacco, the difference between leaf tip and base is much greater, indicating that the rate of cell division varies much more from tip to base in tobacco than in cotton.

While the uniform distribution of sector frequency in irradiated cotton leaves indicates that the frequency of cell division is relatively uniform during leaf development, the relatively uniform size of the sectors in irradiated cotton leaves suggests that the duration of cell division also varies minimally throughout the lamina (Fig. 5). Cell division in tobacco continues at the base of the leaf for six cell cycles after the tip has ceased dividing (R. S. Poethig, unpublished observations) whereas cell division at the base of the cotton leaf ceases approximately three cell divisions after arrest at the tip. Because sector size does not appear to differ significantly across the width of the leaf, it is likely that cell division ceases simultaneously across the width of the leaf.

The regulation of leaf shape

In most, if not all plants, leaves produced early in the growth of the shoot are different in shape to those produced later. This developmental variation in leaf shape occurs to varying degrees in different species. A dramatic example of this phenomenon is found in phyllodineous *Acacia* species, where early leaves are pinnate and late leaves are entire. Goebel coined the term heteroblasty to describe leaf development in such plants, and proposed that this transition could be ascribed to developmental arrest at certain stages along a developmental path (Goebel, 1900). Kaplan (1980) has reinvestigated the development of the phyllode of four *Acacia* species and concluded that Goebel's interpretation of heteroblasty in this group is a misleading simplification. The developmental basis of leaf development is still unclear and, considering the number of factors that affect this phenomenon, is likely to be quite complex.

In *G. barbadense* L., the first leaf produced by the shoot is cordate or very slightly lobed, and successive leaves become increasingly lobed until the shoot begins to produce a 'climax' leaf shape (Fig. 6). Genetic analyses of leaf shape variation in different *Gossypium* species suggest that much of the natural variation in the

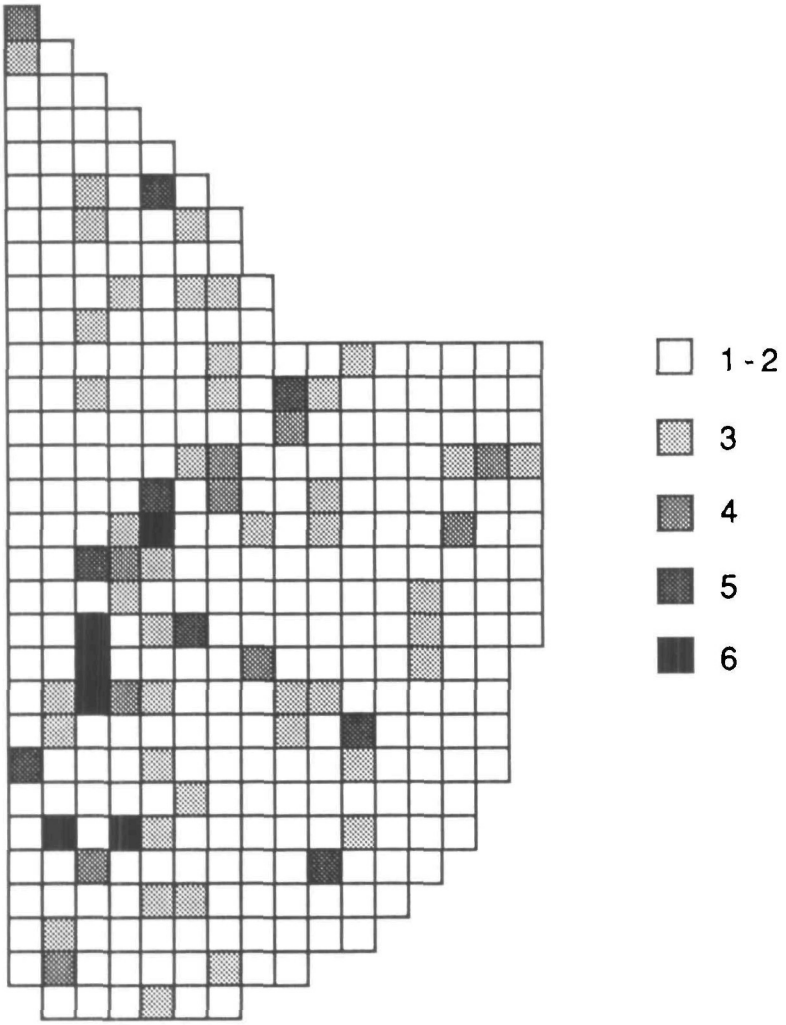

Fig. 4. The distribution of average sector frequency in a leaf irradiated at approximately 1 mm. The numbers represent the actual number of sectors in 25 mm^2.

climax leaf shape within this genus is regulated by alleles of one or two genes that primarily affect the depth of leaf lobes (Stephens, 1944*a*,*b*,*c*). Flowering is another factor which seems to play an equally important role in regulating climax leaf shape (Stephens, 1944*c*). Early flowering genotypes go through the series of leaf shape changes more quickly than the later flowering genotypes and consequently adopt a characteristic climax leaf shape earlier in shoot development (lower node position) than late-flowering varieties. In addition to these genetic mechanisms, environmental conditions play an important role in the development of shape in the cotton leaf. For example, Stephens (1944*c*) showed that it was possible to reveal the full effect of certain alleles by prolonging the vegetative growth of the shoot.

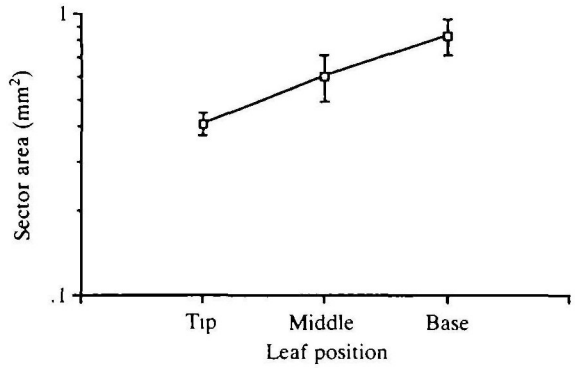

Fig. 5. Mean sector area at three points along the length of a leaf irradiated at approximately 1 mm.

Fig. 6. Heteroblasty in cotton. Leaf 1 (left) and climax leaf (right).

Allometric analysis of leaf growth

The analysis of shape change in any organism is confounded by the fact that unless growth is isometric, i.e. equal in all dimensions, the shape of a structure changes constantly throughout its growth in size. This problem may be obviated by graphing the two dimensional growth of a structure on log–log plots, a method known as allometric analysis (Huxley, 1932). This approach often yields a straight line whose formula,

$$\log y = k\log x + \log b,$$

contains two constants that are useful for describing the growth patterns. k, the allometric constant, is the slope of the line and represents the ratio of the relative growth rates in these dimensions. b represents log y when x=1, that is, the relationship between these dimensions at a single point in development. Assuming that the unit of measurement is small, b generally represents the initial shape of the structure.

Allometric analysis has been used to characterize leaf growth in a number of different species, including *Antirrhinum majus* L. (Harte, 1979, 1983, 1985; Harte and Meinhard, 1979*a*,*b*, 1980), cotton (Hammond, 1941*a*,*b*) and *Ipomoea caerulea* (Njoku, 1957). In *A. majus* L., Harte and co-workers found that the growth in length and width of the developing leaf can be represented by two allometric lines drawn through a scatter of points of the growth data (length *versus* width in these experiments). Early in leaf development the slope of the line relating the length and width is significantly different from the slope of this line later in development. These studies also showed that the successive leaves produced by a shoot differ in shape and that this shape difference can be attributed to increased values of b up the stem (Harte and Meinhard, 1979*a*). This suggests that the heteroblastic differences in leaf shape in *A. majus* are the result of developmental differences occurring in the early primordium. The value of the allometric constant (k) of the length–width relationship in narrow leaf mutants is greater than in wild-type. This indicates that the mutant leaf shape is brought about by decreased growth in width throughout leaf development and is not merely due to a decreased size of the young primordium. Environmental factors such as photoperiod and temperature affect the value of the slope in each mutant in a slightly different way (Harte and Meinhard, 1979*b*). These results reveal how environmental factors (temperature and photoperiod) interact with the developmental age of the shoot to influence leaf development.

In cotton, alleles of the *Okra* locus affect the dissection of the leaf blade by altering lobe length, sinus length, and the width of the leaf blade (Fig. 7). Hammond's (1941*a*,*b*) allometric analysis of the developmental basis of these effects revealed that mutants that produce highly dissected leaves do so by affecting the early differentiation of the primordium in *G. hirsutum* and *G. arboreum*. The L_2^o allele alters the development of the early leaf primordium by (1)

Fig. 7. Phenotype of haploid L_2^o (left) and l_2 (right) cotton leaves. The leaf on the right is hemizygous for the v_7 gene and is light green.

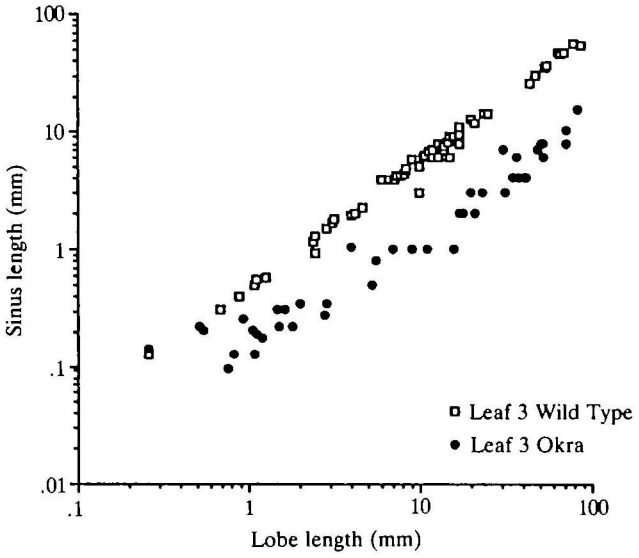

Fig. 8. The relationship between length of the main lobe and length of the sinus during the development of the 3rd leaf of l_2l_2 and $L_2^oL_2^o$ cotton. Data are plotted on logarithmic axes.

delaying the appearance of lateral lobes in the primordium, (2) increasing the rate of lobe growth relative to sinus growth in the primordium, (3) reducing the width of lobe primordia and (4) increasing the rate of lobe elongation relative to the rate of growth in lobe width early in the development of the primordium.

Our analysis of L_2^o in *G. barbadense* L. confirms these observations (Fig. 8). During the growth of the third leaf of wild-type (l_2) plants, the growth of the central lobe and the major sinus exhibits a linear allometric relationship with a slope of 1. The relationship between lobe and sinus growth in L_2^o leaves appears to have two discrete phases. Early in development, lobe length increases more rapidly than sinus length. At a length of about 5 mm, the leaf adopts an isometric growth pattern (k=1.08; R^2=0.92) that parallels the growth of wild-type leaves. This pattern of growth is identical to that which Hammond (1941*a*, 1941*b*) observed for L_2^o in *G. hirsutum*, and confirms her conclusion that L_2^o primarily affects patterning processes during leaf initiation.

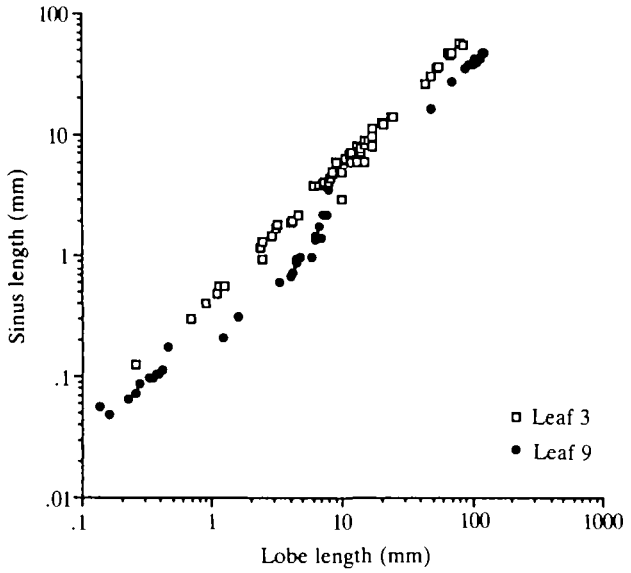

Fig. 9. The relationship between length of the major lobe and length of the sinus during development of the 3rd and 9th leaves of wild-type cotton. Data are plotted on logarithmic axes.

Fig. 9 is a plot of the length of the central leaf lobe against the length of the primary sinus during the growth of third and ninth leaves of wild-type cotton. These curves resemble those representing the development of successive leaves of *A. majus* L. and *I. caerulea* (Harte, 1979; Njoku, 1957). Although the slope of the growth curves of successive leaves in cotton is slightly different, they differ more significantly in their b values. Leaves 3, 4 and 9 have k values of 1.06, 1.07 and 1.03, respectively, for log lobe length *vs.* log sinus length. These values close to 1 indicate that shape does not change throughout leaf development. The b values for these leaves are −0.33, −0.34 and −0.57, respectively, indicating that these primordia are different in shape at the initiation of the analysis. R^2 values for these curves were all greater than 0.98. Thus, heteroblastic differences in cotton leaf shape are determined very early in the development of the primordium, as they are in *A. majus* L. Since lobe length and sinus length grow isometrically (slope=1) in *G. barbadense* L., the shape established during this early phase remains constant throughout the rest of development. A number of other parameters also exhibit variation in their b values at different nodes (Hammond, 1941*a*).

Mosaic analysis

Genetic mosaics are useful in determining the cell autonomy of traits and in determining the primacy of certain cell or tissue types in a variety of biological processes (Hotta and Benzer, 1972; Hake and Freeling, 1986). In plants, genetic mosaics often arise spontaneously as a consequence of somatic mutations, or may be produced by a variety of horticultural and genetic techniques (Stewart and Dermen, 1975, 1979; Hake and Freeling, 1986; Poethig, 1987, 1988). This analysis is facilitated in cotton by the existence of (1) a variety of leaf shape mutations, (2) cell marker mutations that permit the detection of genetic mosaics, such as virescent mutations, which produce pale green or late greening phenotypes and (3) *Semigamy (Se)*, a genetic trait that produces maternal haploid, paternal haploid and mosaic maternal:paternal haploid offspring at a variable frequency (Turcotte and Feaster, 1963, 1967; Endrizzi *et al.* 1984; Stelly and Rooney, 1989). The mechanism of this phenomenon has not been unequivocally established, but probably results from the failure of the sperm nucleus to fuse with egg nucleus during fertilization.

In order to determine if the affect of L_2^o on leaf shape is mediated by diffusible factors or cell-limited factors, we created mosaics composed of wild-type (l_2) and L_2^o tissue. A pale green (v_7v_7), Semigamy (*SeSe*), wild-type (l_2l_2) stock was crossed by a dark green (V_7V_7), Okra ($L_2^oL_2^o$), non-Semigamy (*sese*) male parent. Approximately 3% of the progeny of this cross were maternal or paternal haploids, and approximately one quarter of these were chimeric for maternal and paternal tissue. Sectorial chimeras derived from this cross suggest that the L_2^o trait is cell autonomous. In leaves bisected by a sector boundary, the phenotype of each region corresponded strictly to its genotype even when tissue of a particular genotype was present in only a small portion of the leaf. In the sectorial leaf shown in Fig. 10, the two dark green lateral lobes express the narrow lamina characteristic of L_2^o, whereas the pale green central lobe expresses the l_2 phenotype. In the region of lamina that has an intermediate phenotype (arrow), the lower cell layers of lamina are L_2^o and the upper layers of the lamina are l_2. Thus, lateral diffusion of L_2^o factors does not occur, even over relatively short distances. It should be noted that we cannot be certain that this gene is completely cell autonomous because L_2^o does not have an obvious cellular phenotype.

Conclusions

The aim of our work is to describe cellular parameters of leaf growth in cotton and to obtain a greater understanding of how the regulated, progressive changes in leaf shape (heteroblasty) take place. In addition we have investigated the effect of the L_2^o mutation on leaf development.

Although the overall pattern of cell division in the cotton leaf is superficially similar to that of tobacco, there are some striking differences. Cessation of cell division takes place over a long period in tobacco (5–6 cell cycles). In cotton, on the other hand, the time between the cessation of division at the tip and the base is no more than three cell cycles. The second and most striking difference is that the orientation of marginal cell divisions is different in cotton than tobacco. In tobacco, most sectors induced at the margin late in development run parallel to the margin, indicating that the marginal cells contribute little to the growth of the leaf lamina. In contrast, marginal sectors in cotton are

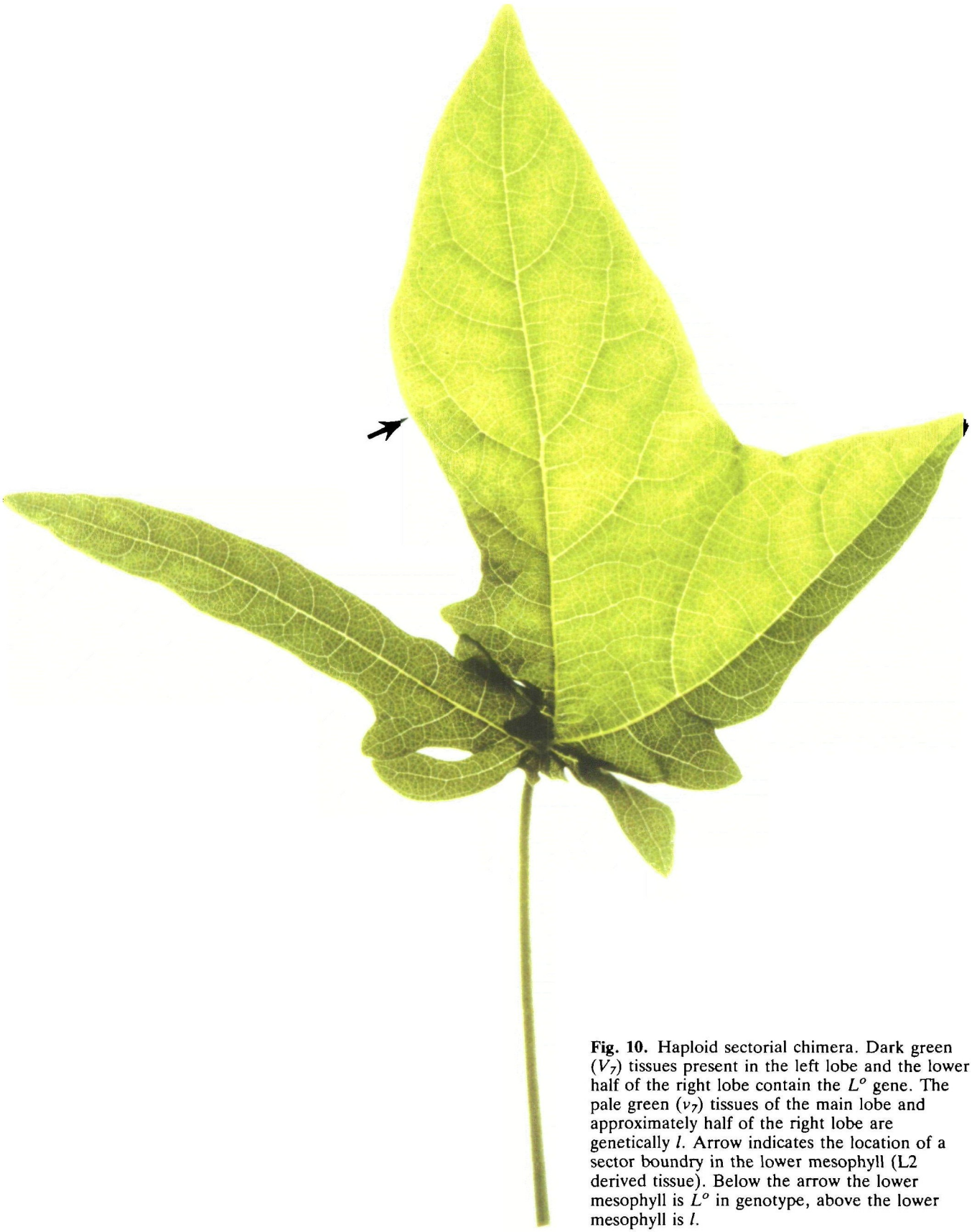

Fig. 10. Haploid sectorial chimera. Dark green (V_7) tissues present in the left lobe and the lower half of the right lobe contain the L^o gene. The pale green (v_7) tissues of the main lobe and approximately half of the right lobe are genetically l. Arrow indicates the location of a sector boundry in the lower mesophyll (L2 derived tissue). Below the arrow the lower mesophyll is L^o in genotype, above the lower mesophyll is l.

often elongated perpendicular to the edge of the lamina indicating that they do give rise to a significant portion of the leaf blade. However, the area of these sectors falls within the range of sector areas in the intercalary portion of the leaf, suggesting that the marginal cells do not contribute disproportionately to the growth of the lamina.

The frequency of sectors in the lamina indicates that there are no localized regions with significantly different rates of cell division in the leaf after it is approximately 1 mm in length. This observation is corroborated by allometric analysis which suggests that the pattern of wild-type leaf growth is laid down early in development.

The allometric analysis of the relative growth of the leaf suggests that heteroblastic change is attributable to changes that occur in the very young primordium. This is also the case in *A. majus* L. All leaves adopt a k value of approximately 1 after they reach a length of 0.2 to 0.3 mm, suggesting that the shape of the primordium does not change after this time. Allometric analysis of leaf 3 on L_2^o plants suggests that this mutation causes the early relative growth rate observed for wild-type leaves to be extended until the leaf is 3–5 mm in length. This extension of an early growth pattern in combination with the delayed initiation of lateral lobes probably accounts for the effect of this mutation, because later in development mutant and wild-type leaves have the same relative growth rate. This suggests that the wild-type l_2 gene may play a role in heteroblastic development in cotton.

The autonomous expression of L_2^o in sectorial leaves, in combination with the demonstration of the early effect of the mutation, suggest that this gene is expressed within the leaf primordium. The way in which this leaf-specific factor interacts with external factors that regulate heteroblastic development could shed some light on the regulation of heteroblasty in cotton.

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