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The control of trichome spacing and number in *Arabidopsis*

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SUMMARY

Arabidopsis **trichomes are single-celled epidermal hairs that serve as a useful model for the study of plant cell differentiation. An examination of the distribution of trichomes early in their development revealed that developing trichomes occur adjacent to another trichome much less frequently than would be expected by chance. Clonal analysis of epidermal cell lineages ruled out a role for cell lineage in generating the observed minimum-distance spacing pattern. Taken together, these results are consistent with a role for lateral inhibition in the control of trichome development. We also report the identification of a new locus, *Reduced Trichome Number (RTN),* which affects the initiation of trichomes. This locus was initially detected by the reduced number of leaf trichomes on Landsberg *erecta* plants compared to that on Columbia plants. Quantitative Trait Locus mapping revealed that more than 73% of the variation in trichome number was due to a major locus near *erecta* on chromosome 2. The reduced number of trichomes conditioned by the Landsberg *erecta* allele of this locus appeared to be due to an early cessation of trichome initiation. The implications of these observations are discussed with regard to previously published models of trichome development.**

Key words: *Arabidopsis*, trichome, epidermis, leaf development, clonal analysis, QTL mapping, *RTN* locus, pattern formation, stomata

INTRODUCTION

A common phenomenon occurring during the development of many organisms is the differentiation of a specialized cell type from a field of otherwise equivalent cells. Examples include the formation of bristles in the insect epidermis (Artavanis-Tsakonas et al., 1995; Lawrence and Hayward, 1971; Wigglesworth, 1940), the formation of nitrogen-fixing heterocysts in filaments of the blue-green alga *Anabaena* (Wilcox et al., 1973), and the formation of stomata in the plant epidermis (Sachs, 1978). In principle, the differentiated elements could be located randomly within the field of surrounding cells. However, the arrangement of elements in most naturally occurring structures exhibits some degree of order. The simplest pattern observed is the maintenance of a minimum distance between neighboring elements, which is often called a 'spacing pattern' (Wolpert, 1971; Wolpert and Stein, 1984).

Due to their simplicity, spacing patterns in two-dimensional fields of cells have played an important historical role in stimulating theoretical efforts to model pattern formation (Wolpert and Stein, 1984). Such models generally invoke an autocatalytic activator that acts over a short range, and a rapidly diffusing inhibitor acting over a longer range (Turing, 1952; Meinhardt and Gierer, 1974). However, little is known regarding the mechanistic basis for most patterns observed in real organisms. Perhaps the best characterized example is the patterning of sensory bristles in the epidermis of *Drosophila*. Bristle formation is promoted by transcription factors of the *acheate-scute* complex, while neighboring cells are prevented from becoming bristles via inhibitory signaling ('lateral inhibition') mediated by the gene products of *Notch* and *Delta* (reviewed by Artavanis-Tsakonas et al., 1995). The spacing of *Anabaena* heterocysts also appears to be regulated by an inhibitory signal originating from newly specified heterocysts (Wilcox et al., 1973). In contrast, the minimum distance between stomata in the epidermis of several dicotyledonous plant species appears to be established largely by a stereotyped cell lineage pattern associated with stomatal differentiation (Sachs, 1978), although inhibitory interactions may also play a role in some plant species (Kagan et al., 1992; Korn, 1993).

The formation of hairs (trichomes) in the epidermis of *Arabidopsis thaliana* provides another example of the differentiation of a particular cell type within a field of equivalent cells (reviewed by Marks, 1994). *Arabidopsis* trichomes are highly specialized single cells that are expanded out of the plane of the epidermis. On leaves, trichomes are generally branched. Trichomes begin to form on the adaxial surfaces of leaves very early in leaf development, at a time when the developing epidermis (protoderm) is still dividing rapidly. The first detectable sign of trichome initiation is an increase in both cell and nuclear size. This is followed by the elongation of the cell perpendicular to the plane of the leaf, the elaboration of

branches, and the maturation of the characteristic trichome cell wall. The initiation of trichomes proceeds basipetally, with the first trichomes forming near the distal end of the developing leaf. As trichomes begin to develop on more basal regions of the leaf primordium, new trichomes continue to form between the maturing trichomes on the distal part (Hülskamp et al., 1994; Marks, 1994). In the mature leaf, trichomes are distributed relatively uniformly, with adjacent trichomes occurring only rarely (Hülskamp et al., 1994; Larkin et al., 1994).

Several investigators have focused on trichome development as a model for studying cell differentiation in plants (Oppenheimer et al., 1992; Hülskamp et al., 1994). A number of mutations that affect *Arabidopsis* trichome development have been isolated (Lee-Chen and Steinitz-Sears, 1967; Feenstra, 1978; Koornneef et al., 1982; Haughn and Somerville, 1988; Hülskamp et al., 1994; Marks and Esch, 1994). These genetic analyses have identified five genes involved in the early events of trichome development. Mutations in either of two genes, *GLABRA1* (*GL1*) or *TRANSPARENT TESTA GLABRA* (*TTG*), eliminate virtually all trichomes from the shoot epidermis (Koornneef et al., 1982). The *GL1* gene encodes a protein with sequence similarity to the DNA-binding domain of the MYB family of transcriptional activators (Oppenheimer et al., 1991). *GL1* transcripts are present at a low level throughout the protoderm, with much higher levels of expression in developing trichomes and presumptive trichome precursor cells (Larkin et al., 1993). These results are consistent with the results of genetic mosaic experiments demonstrating that *GL1* acts locally (Rédei, 1967; Hülskamp et al., 1994). The *TTG* gene has not yet been isolated. However, recent results indicate that expression of the maize *R* gene in *ttg* mutant plants results in functional complementation of the *ttg* mutation (Lloyd et al., 1992). This observation suggests that *TTG* may encode a homolog of the maize *R* gene, although it remains possible that *TTG* merely regulates the expression of an *R* homolog. The maize *R* gene encodes a protein with sequence similarity to the helix-loop-helix family of transcriptional activators (Ludwig et al., 1989). Based on the results of epistasis analysis between *gl1* and *ttg* mutations and transgenic constructs constitutively expressing *GL1* and *R*, it was proposed that *GL1* and *TTG* cooperate at the same point in trichome development (Larkin et al., 1994). This study also demonstrated that reducing the level of functional *TTG* can result in clusters of adjacent trichomes on leaves. Mutations at the *TRYPTICHON* (*TRY*) locus also result in trichome clustering (Hülskamp et al., 1994). These results have led to the proposal that committed trichome precursor cells send an inhibitory signal to adjacent cells, preventing them from differentiating as trichomes (Hülskamp et al., 1994; Larkin et al., 1994).

In this study, we have examined the spacing of trichomes early in leaf development, and have found that they are initiated adjacent to other trichomes much less frequently than would be expected by chance. Fate mapping of trichomes and their associated group of accessory cells using transposon-induced clonal sectors provides no evidence for a special cell-lineage pattern associated with their development. We also describe a new locus, *REDUCED TRICHOME NUMBER* (*RTN*), which affects the number of leaf trichomes This locus was detected as a variant between the Columbia (Col) and Landsberg *erecta* (Ler) ecotypes.

MATERIALS AND METHODS

Plant strains and growth conditions

The Col and Ler strains supplied with the recombinant inbred (RI) lines were used for the observations reported here. Linkage of low trichome number to the *erecta* locus also has been observed in crosses between Col-0 and Ler strains obtained from G. Redéi (University of Missouri, Columbia, MO) and maintained in our laboratory. The Lister and Dean RI lines (Lister and Dean, 1993) were obtained from the Nottingham *Arabidopsis* Stock Center. A transgenic derivative of Ler containing a 35S-β-glucuronidase gene interrupted by an *Ac* transposon inserted into the 5′ untranslated leader (35S*GUS*::*Ac*; Lawson et al., 1994) was supplied to us by C. Dean (John Innes Center, Norwich, UK). Data for the genotypes of the RI lines were obtained from the *Arabidopsis thaliana* Database (http://weeds.mgh.harvard.edu).

Plants were grown on one to two inches of a 3:1 mixture of fine vermiculite (Peters) and African Violet soil (Hyponex) layered on top of coarse vermiculite in a growth chamber maintained at 22°C under constant illumination with fluorescent lights (200 μE m^{-2} $seconds^{-1}$). The soil was watered initially with a complete nutrient solution (Feldmann and Marks, 1987).

Microscopy and developmental analysis

The first two leaves of *Arabidopsis* are initiated more or less simultaneously and cannot be distinguished from one another. Therefore, throughout this work, the term ‘first leaf’ is used to refer to either one of the first two leaves. For the measurement of parameters related to trichome spacing, whole-mount preparations were examined with a Nikon Diaphot 200 microscope with DIC optics. Seedlings 4-5 days postgermination were mounted in an 8:1:2 w/v/v mixture of chloral-hydrate/glycerol/water (Berleth and Jurgens, 1993) without fixation and cleared for 15 minutes. Seedlings with first leaf primordia between 140 μm and 200 μm in length were selected for examination. At this point in development, trichome initiation has proceeded approximately two-thirds of the distance from the tip of the leaf towards the base. The cross hairs of an ocular micrometer were used to delimit a line crossing the primordium at right angles to the axis of the leaf, two cell diameters proximal to the basal-most developing trichome, and all cells distal to this line were counted. For the comparison of trichome development in Col, Ler, and CL67 plants, whole mounts were made at daily intervals. In addition to recording the number of trichomes on each leaf primordium, leaf length and leaf width were measured with an ocular micrometer.

Selection of L1 sectors

Plants containing the 35S*GUS*::*Ac* gene were grown until the first two leaves were fully expanded (approximately 2 weeks), and then prefixed under vacuum in 0.1% formaldehyde, 100 mM $NaPO_4$, pH 7.0, 0.1% Triton X-100 for 15 minutes. After rinsing twice with 50 mM $NaPO_4$, pH 7.0, the plants were immersed overnight in 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 50 mM $NaPO_4$, pH 7.0, 1 mM $K_3Fe(CN_6)$, 0.1% Triton X-100 (Jefferson et al., 1987; De Block and Debrouwer, 1992) at 37°C, and then fixed and cleared as described previously (Larkin et al., 1993).

The epidermis is derived from the outermost layer of the apical meristem, which is known as the L1 layer. Seven L1 sectors that ran from the base of the petiole to the distal margin of first leaves were selected for analysis. L1 sectors were recognized by the staining of trichomes and guard cells. Examination of hand-cut sections confirmed that the sectors were exclusively in the L1. One L2 sector that ran the full length of the leaf and invaded the L1 in the distal portion of the leaf also was used in the analysis. Sector width was estimated by the fraction of the petiole width occupied by the sector. All but one of the sectors used in this study occupied approximately one-fifth to one-sixth of the petiole width. The remaining sector occupied approximately one-half of the petiole width. No narrower sectors extending the full length of the leaf were observed.

Mapping number of trichomes per leaf as a quantitative trait

To map loci associated with reduced number of trichomes per leaf, the number of trichomes on the first leaf of ten plants was counted for each of 99 recombinant inbred (RI) lines derived from a cross between Col and Ler by Lister and Dean (1993). The mean number of trichomes per leaf was calculated, and these phenotypic values were compared with DNA marker data for 67 loci spread throughout the *Arabidopsis* genome, using the computer program Mapmaker-QTL (Lander and Botstein, 1989). Although Mapmaker-QTL was originally written for F_2 and backcross populations, it has been shown to be sufficiently robust for the analysis of recombinant inbred populations (Wang et al., 1994). Any genomic region with a LOD (Log of Odds Ratio) score greater than 2.5 was considered to be a putative trichome number locus. LOD is defined as the Log_{10} of the ratio between the odds in favor of the existence of a significant locus at a particular point on the genetic map versus the odds that no locus is present. This level for declaring significant loci gives an experiment-wide probability for false positives of approximately 0.05 (Lander and Botstein, 1989). The data for the number of trichomes per leaf in the RI lines are available from the authors upon request.

RESULTS

The trichome distribution on *Arabidopsis* leaves is not random

Inspection of the pattern of trichomes on mature *Arabidopsis* leaves suggests a greater degree of uniformity than would be expected from a random distribution (Fig. 1A). This impression was confirmed by the application of a standard measure of the degree of randomness present in a spatial pattern. The parameter R is the ratio of the measured average distances between nearest neighbors and the average nearest neighbor distance expected for a random pattern with the same density (Clark and Evans, 1954). A completely random distribution would have a value of R=1, while a completely ordered hexagonal pattern would have a value of R=2.15. For *Arabidopsis* trichomes of the Col ecotype, R=1.40, a significant deviation from the expectation for a random distribution ($P<0.01$). This value of R is similar to the R values obtained for minimum distance spacing patterns such as the distributions of stomata on the leaves of dicots (Sachs, 1978) and hairs on the epidermis of insects (Wigglesworth, 1940; Lawrence and Hayward, 1971).

However, the distribution of trichomes on mature leaves does not necessarily reflect the distribution at the time of their formation. The initiation of new trichomes ends before the cessation of cell divisions in the leaf epidermis, and therefore cell divisions occurring after the formation of the last trichomes contribute to the distance between adjacent trichomes on the mature leaf. Because our primary interest was in the role of trichome spacing during the initiation of trichome development, it was necessary to examine their spacing at the time of initiation. Previous studies have noted that developing trichomes are separated on average by three to four cells (Hülskamp et al., 1994; Larkin et al., 1994), but the regularity of this spacing has not been rigorously investigated.

Trichome spacing was examined on the adaxial surface of first leaves of Col plants when the leaf primordia were between 140 μm and 200 μm in length (Fig. 2A). At this stage, trichome development has proceeded approximately two-thirds of the distance from the tip of the leaf towards the base. All adaxial protodermal cells in this region of each primordium were counted and all cells recognizable as developing trichomes were counted (Table 1). In a sample of 2,120 cells from 18 leaf primordia, 79 developing trichomes were observed. Based on the density of developing trichomes and the average number of cells neighboring a typical protodermal cell, approximately 17 of these trichomes would have been expected to neighbor another trichome, assuming a random distribution (Table 1). In fact, none of these developing trichomes were adjacent to another trichome. The probability of obtaining no trichomes neighboring another trichome from this sample if the distribution were random is less than 10^{-8} (Table 1). This observation indicates that a minimum distance between developing trichomes is established at the time of their initiation.

Cell lineage does not play a role in trichome spacing

Sachs and coworkers have shown that the specialized cell lineage associated with stomatal development is responsible for much of the observed stomatal spacing pattern in many plant species (Marx and Sachs, 1977; Sachs, 1978). In most dicots, the first sign of stomatal development is the formation of a small, triangular stomatal mother cell by an unequal division of a protodermal cell (Esau, 1977). The stomatal mother cell undergoes a series of divisions, the net result of which is a pair of guard cells surrounded by ordinary epidermal

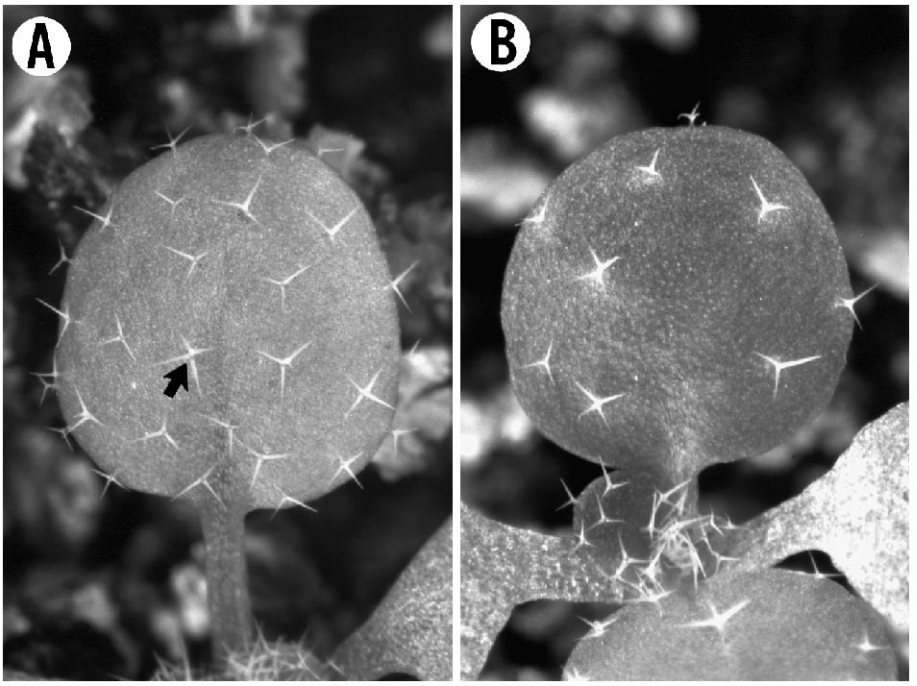

Fig. 1. Trichome distribution on first leaves of Col and Ler plants. (A) Col. (B) Ler. The arrow in A indicates a typical trichome.

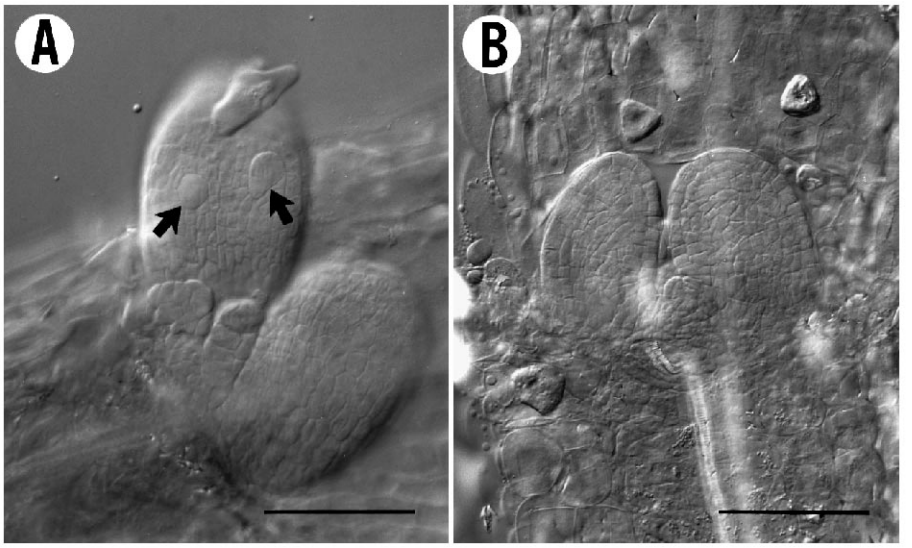

Fig. 2. Trichome development on *Arabidopsis* leaf primordia. (A) Col first leaf primordium with three developing trichomes. Arrows, newly initiated trichomes. (B) Col leaf primordium prior to the initiation of trichome development. Scale bars, 100 μm.

cells, all of which are descendants of the mother cell. The existence of this lineage unit appears to explain the minimum distance of approximately one epidermal cell that is maintained between stomata (Sachs, 1978), although the pattern may be fine-tuned by interactions between developing stomatal mother cells (Kagan et al., 1992). There is no evidence for such a specialized pattern of cell divisions associated with trichome development; however, trichomes are surrounded by 10-12 accessory cells that appear morphologically specialized, and it is difficult to rule out the possibility, by anatomical methods, that these cells are the result of a specialized lineage directly associated with trichome differentiation.

Genetically marked clonal sectors resulting from the excision of an *Ac* transposon from a β-glucuronidase (*GUS*) transgene (Dolan et al., 1994; Lawson et al., 1994; Scheres et al., 1994) were used to investigate the role of cell lineage in determining trichome spacing. Excision of the *Ac* element from the 35S*GUS* gene in a cell results in the expression of the GUS enzyme in all descendants of this cell. Histochemical staining results in the formation of a blue precipitate, which allows GUS expression to be localized.

If a specialized cell lineage analogous to the stomatal lineage pattern plays an important role in preventing neighboring protodermal cells from becoming trichomes, then each trichome should be surrounded by cells that are its closest clonal relatives. A prediction of the lineage model of spacing is that if the trichome is included in a sector representing a cell lineage originating well before the trichome was formed, then all or most of the associated accessory cells should also be included in the sector. In this case, if a trichome was located near the sector boundary, the boundary would be expected to 'detour' around the trichome and include the accessory cells. However, if no lineage constraint on the relationship between trichomes and their accessory cells exists, then sector boundaries should exhibit no special relationship to the accessory cells.

To ensure that the sectors used for clonal analysis of trichome development represent lineages initiated well before the start of trichome development, GUS-expressing sectors that ran the full length of the proximal-distal axis of the leaf (Fig. 3A) were selected. All sectors of this type encompassed at least one-sixth of the width of the adaxial epidermis, consistent with published scanning electron micrographs that show that the adaxial epidermis of newly initiated first leaves is composed of five or six files of cells (Irish and Sussex, 1992; Medford et al., 1992). These sectors represented excision events that occurred in founder lineages of the epidermis, several cell divisions prior to the onset of trichome development (see Discussion).

Table 1. Analysis of trichome spacing in the adaxial protoderm of first leaves of *Arabidopsis* ecotype Col

Number of leaf primordia	18[a]
Total number of cells	2,120[b]
Total number of trichomes	79
Number of trichomes adjacent to another trichome	0
Mean number of neighboring cells per randomly chosen cell (n)	6.2±0.1[c]
Fraction of cells that are trichomes (x)	0.041
Fraction of cells that are not trichomes (1-x)	0.959
Probability that no neighbors of a given cell are trichomes $(1-x)^n$	0.79
Probability that at least one neighbor of a given cell is a trichome (1-0.79)	0.21
Expected number of trichomes adjacent to another trichome	16.6[d]
Probability that no trichomes in this sample are adjacent to another trichome	8.2×10^{-9}[e]

[a]Each leaf was between 140 μm and 200 μm in length and had 1 to 5 developing trichomes on its adaxial surface.

[b]At this stage, developing trichomes are found only on the distal one-half to two-thirds of the leaf surface. All adaxial protodermal cells were counted in a region extending from the distal tip of the leaf to a line transverse to the leaf axis and two cell diameters proximal of the basal-most developing trichome.

[c]The number of neighboring cells was determined for 50 randomly chosen cells in the adaxial protoderm. The data are given as the mean number of neighbors ± s.e.m.

[d]The expected number of trichomes adjacent to another trichome was calculated by multiplying the probability that at least one neighbor of a given cell is a trichome (0.21) with the number of trichomes in the sample (79).

[e]The probability of observing no trichomes adjacent to another trichome in this sample was calculated directly. If the probability that no neighbors of a given cell are trichomes is 0.79, then the probability that 79 cells (which happen to be developing trichomes) have no neighbors that are trichomes is $(0.79)^{79} = 8.2\times10^{-9}$.

The argument presented in this Table is an extension of the reasoning of Fobert et al. (1994) in their discussion of the detection of cell cycle synchrony in meristems.

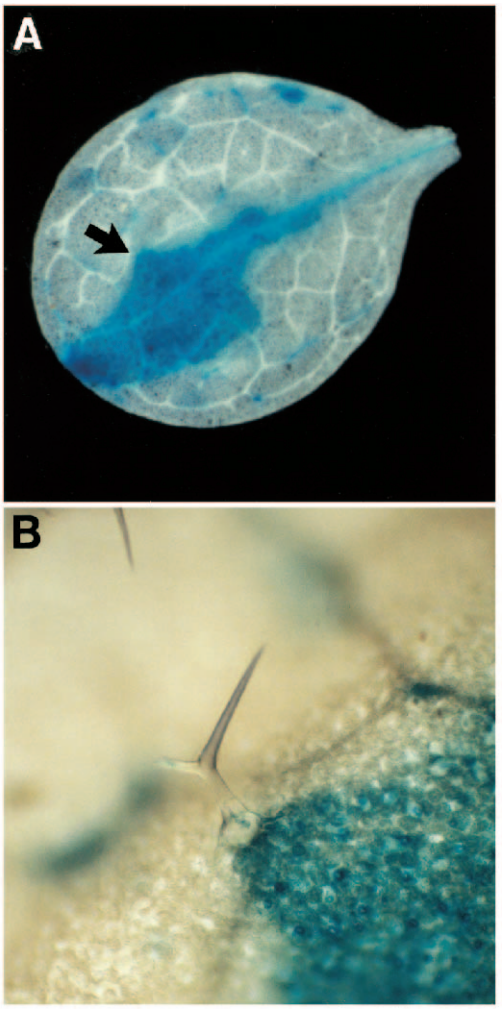

Fig. 3. Clonal analysis of cell lineages associated with trichome development. (A) Early *Ac* excision sector typical of those used for analysis of trichome lineages. This sector occupied approximately one-fifth of the width of the petiole (4 of 21 epidermal cells stained across the width of the petiole). The arrow indicates the position of the Type A trichome sector shown in panel B. (B) A Type A trichome sector (see Fig. 4) with five stained accessory cells. Although the trichome has lost much of its cytoplasm through a broken branch tip, blue staining is clearly visible in the remaining cytoplasm near the base of the trichome.

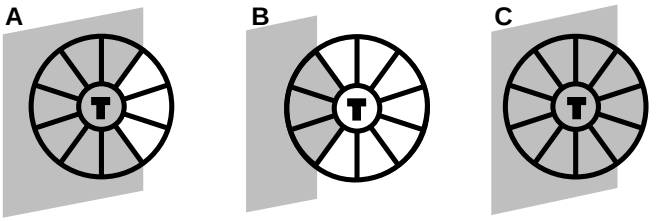

Fig. 4. Relationship between trichomes adjacent to a sector boundary and the sector boundary. Shading indicates GUS-expressing (blue-stained) cells. (A) Type A trichomes. Trichome and some accessory cells stained. (B) Type B trichomes. Trichome not stained. Some accessory cells stained. (C) Type C trichomes. Trichome and all accessory cells stained.

Table 2. Analysis of the staining pattern of trichomes and surrounding accessory cells for trichomes adjacent to the border of Ac-generated clonal sectors

Trichome type	Number of trichomes	Fraction of accessory cells staining
A. Trichome stained, some accessory cells stained	11	0.60[a]
B. Trichome not stained, some accessory cells stained	11	0.32[b]
C. Trichome and all accessory cells stained	3	1.0

Trichomes adjacent to the sector boundaries of five L1 sectors were examined for staining of the trichome and accessory cells.

[a]Number of accessory cells staining/total accessory cells for individual trichomes of Type A: 8/12, 12/16, 2/9, 5/12, 4/9, 6/10, 11/12, 12/15, 6/10, 5/11, 6/12.

[b]Number of accessory cells staining/total accessory cells for individual trichomes of Type B: 2/10, 2/10, 2/9, 2/9, 3/12, 8/12, 6/10, 1/11, 6/12, 2/12, 4/13.

Trichomes were considered to be adjacent to the edge of a sector if at least one accessory cell was stained and at least one accessory cell was adjacent to an unstained cell. These criteria led to the recognition of three types of relationships between a sector boundary and a trichome with associated accessory cells: cases where the trichome and some accessory cells were stained (Fig. 4A), cases where the trichome was not stained and some accessory cells were stained (Fig. 4B), and cases where the trichome and all of the accessory cells were stained (Fig. 4C). Fourteen trichomes adjacent to a sector boundary were identified (Table 2). In six cases, the trichome was included within the sector (Type A), but only some accessory cells were stained (Fig. 3B). In these instances, 60% of the accessory cells were included within the sector. In another six cases, the trichome was not stained (Type B); in these instances, 32% of the accessory cells were included within the sector. Finally, three cases were observed in which the trichome and all of the accessory cells were stained (Type C).

These data provide no support for the existence of a specialized cell lineage associated with trichome differentiation that could generate the observed minimum-distance spacing pattern. Although approximately twice as many accessory cells were stained in Type A trichomes as were stained in Type B trichomes, a wide variation in the proportion of accessory cells stained was observed among individual trichomes of each type (Table 2, footnotes [a] and [b]). For example, one Type A trichome had only two stained accessory cells out of a total of nine, while one Type B trichome had eight stained accessory cells out of a total of twelve. This degree of variability in the fraction of stained accessory cells is difficult to reconcile with the existence of a highly organized cell lineage.

Particularly telling is the observation that only three of the 25 trichomes adjacent to a sector border were of Type C. If each developing trichome were surrounded by its immediate clonal siblings, as predicted by the cell lineage model of spacing, then most trichomes adjacent to a sector boundary should be of Type C. The low number of Type C sectors is, however, consistent with the absence of a lineage constraint on the relationship between trichomes and their accessory cells. There are more ways for a sector boundary to pass among a group of cells (Type A and Type B) than there are for a boundary to be tangent to a group of cells (Type C).

For comparison, a similar analysis of stomatal lineages was conducted. A typical pair of guard cells contacted 3.6±0.1 ordinary epidermal cells. Thirty stomata adjacent to a sector boundary were examined, with guard cell pairs taking the place of trichomes, using the same criteria for classifying the type of stoma. Twenty-three stomata (77%) were completely surrounded by stained epidermal cells, and were thus equivalent to the Type C class of trichomes described above. Four Type A stomata (13%) were observed. All of these had only a single unstained cell adjacent to a guard cell pair. Three Type B stomata (10%) were observed. All of these had only a single stained epidermal cell adjacent to a guard cell pair. As expected, clonal analysis supports the existence of a specialized cell lineage surrounding the stomata. The existence of a number of Type A or Type B stomata in this sample indicates that while the cell lineage associated with stomatal development contributes to the stomatal spacing pattern, stomata are not always isolated from unrelated neighboring cell lineages.

Identification of a new locus affecting trichome number

In the course of these studies, it was noted that plants of the Ler ecotype have significantly fewer trichomes per leaf than Col plants (Fig. 1B, Fig. 5, Table 3). When Ler and Col plants were crossed, the F_1 plants had an intermediate number of trichomes per leaf, suggesting additive gene action (Fig. 5, Table 3). In the F_2 and the Ler×F_1 backcross populations (Fig. 5, Table 3), plants homozygous for the *erecta* (*er*) mutation derived from the Ler parent had significantly fewer trichomes than did ER^+ plants (Fig. 5, Table 3). These results suggested that a major locus affecting trichome number was linked to *er*.

To investigate the genetic basis of this difference in trichome number, the mean number of trichomes per first leaf was determined for the series of recombinant inbred (RI) lines generated by Lister and Dean (1993) from a cross between Col and Ler (Fig. 6). This analysis confirmed the presence of a major locus controlling trichome number in the interval between *er* and the marker m220 on chromosome 2. This locus had a LOD score of 27.54 and accounted for more than 73% of the total variation in trichome number. In terms of gene action, the locus was partially additive, with a tendency toward dominance (data not shown). This locus is separable from *er*; some ER^+ RI lines with cross-overs in the *ER*-m220 interval produced very low numbers of trichomes (Table 4, CL386), while some *er* mutant RI lines with cross-overs in this interval produced a relatively high numbers of trichomes (Table 4; CL209, CL242, and CL283). No other major loci were detected, although a genomic region on chromosome 3 in the interval between the markers g4523 and m228 had a LOD score of 3.34, and another minor locus on chromosome 4 in the interval between g3843 and g2616 had a LOD score of 2.78. None of these loci mapped near any of the previously identified genes affecting trichome development. We propose that the major locus linked to *er* be named *REDUCED TRICHOME NUMBER* (*RTN*).

Developmental basis of the *RTN* phenotype

Two alternative developmental explanations for the *RTN* phenotype were considered: The RTN^{Ler} allele could result in a lower rate of trichome formation than the RTN^{Col} allele throughout development, or the RTN^{Ler} allele could produce the same initial rate of trichome formation, but result in an earlier cessation of trichome initiation. To distinguish between these possibilities, the initiation of trichomes on the adaxial surface of first leaves of Col and Ler plants was examined at daily intervals throughout development, beginning on the fourth day after planting. When trichome number was plotted against leaf length, it was apparent that early in development (leaf length <200 μm), both Col and Ler leaves produced the same number of trichomes (Fig. 7). Later in development, the number of trichomes produced by Ler leaf primordia dropped

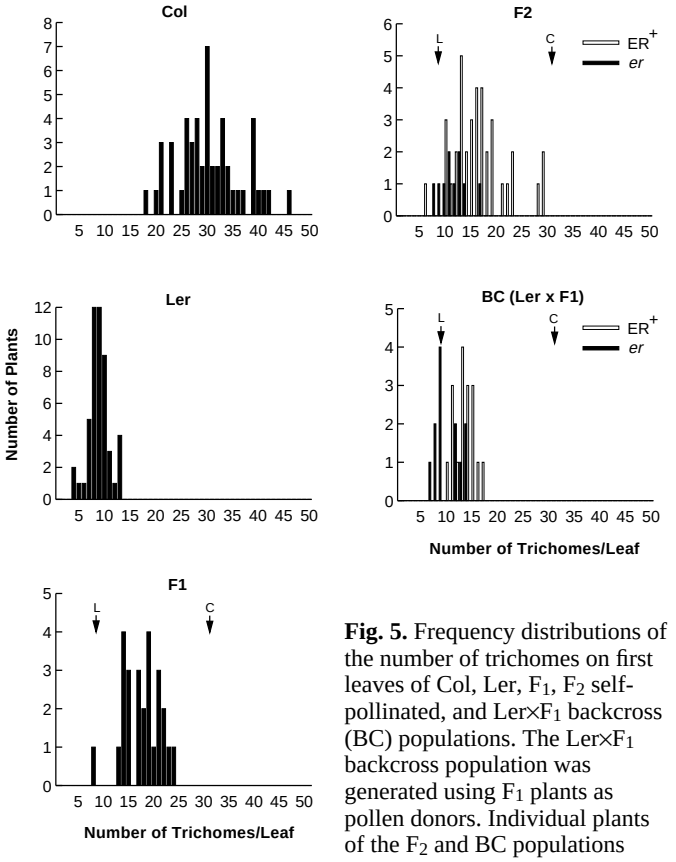

Fig. 5. Frequency distributions of the number of trichomes on first leaves of Col, Ler, F_1, F_2 self-pollinated, and Ler×F_1 backcross (BC) populations. The Ler×F_1 backcross population was generated using F_1 plants as pollen donors. Individual plants of the F_2 and BC populations were scored for the *er* mutation. Arrows indicate the mean number of trichomes per leaf for the Ler (L) and Col (C) populations.

Table 3. Average number of trichomes on fully expanded first leaves of various genotypes and segregating populations

Genotype	Number of trichomes/leaf[a]	Number of leaves examined
Col	30.5±0.9	50
Ler	8.9±0.3	50
Col/Ler F_1	17.6±0.7	26
F2 (self), *er*	11.8±0.8	10
F2 (self), ER^+	16.5±0.9	36
Backcross (Ler×F_1), *er*	10.3±0.7	12
Backcross (Ler×F_1), ER^+	13.6±0.5	18
RI lines, *er*	15.4±0.7	67[b]
RI lines, ER^+	29.5±1.3	33[c]
CL67 (*er* $m220^{Ler}$)	14.4±1.0	10

[a]Mean ± s.e.m. One leaf from the first pair of leaves was examined per plant.
[b]Number of RI lines used to calculate mean.
[c]Number of RI lines used to calculate mean.

off rapidly; by the time Ler leaf primordia are 500 μm long the initiation of trichomes has virtually stopped (Fig. 7). In contrast, Col leaves continue to produce trichomes even after the leaves are 700 μm long (Fig. 7). In the Col background, the initiation of trichomes ceases in each region of the leaf approximately at the time when stomatal mother cells are first detected.

Mature Ler leaves are somewhat different from Col leaves in shape, the most obvious difference being that Ler leaves have shorter petioles (Fig. 1). For this reason, it was important to consider whether these differences had any influence on the phenotype of the *RTN* locus. This seems unlikely. No difference was detected between Col and Ler in the length to width ratio of leaf primordia less than 700 μm long, and the number and size of adaxial epidermal cells also appears to be similar for both ecotypes throughout the period of development examined (J. Larkin, unpublished data). The number of adaxial epidermal cells on mature leaves of Col (Geissler and Sack, personal communication; J. Larkin, unpublished observations) and Ler (Pyke et al., 1991) plants appear to be very similar as well (see Discussion).

To determine if the early cessation of trichome initiation observed with Ler was specifically a phenotype of the *RTN* locus, we examined the production of trichomes in the RI line CL67. The CL67 line has a low trichome number phenotype (Table 3), and has the Landsberg alleles of *er* and m220. However, the majority of the genome in this line is derived from Col; more than 76% of the molecular markers in this line are Col alleles (Lister and Dean, 1993). In particular, the regions from g4523 to m228 on chromosome 3 and from g3843 to g2616 on chromosome 4, where linkage analysis suggested the possibility of minor loci affecting trichome number, are derived from Col. Although the final number of trichomes produced on CL67 leaves was somewhat higher than on Ler leaves (Fig. 7C, Table 3), CL67 showed the same pattern of development. Initially, trichomes were produced at the same rate as on Col leaves, but trichome development ends much sooner on CL67 leaves (Fig. 7).

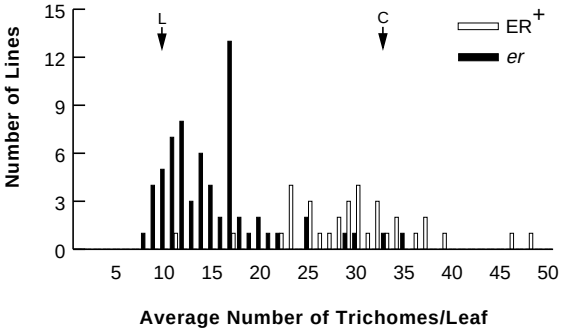

Fig. 6. Frequency distribution of the mean number of trichomes per first leaf of 99 RI lines from the collection of Lister and Dean (1993). For each RI line, the mean number of trichomes per leaf was determined from counts of trichomes on ten first leaves. The *er* genotype of each RI line was scored, and in each case it corresponded to the genotype reported by Lister and Dean (1993) for that line. Arrows indicate the mean number of trichomes per leaf for the Ler (L) and Col (C) populations grown in the same flats as the RI lines.

Trichomes are not produced until leaf primordia are approximately 100 μm long

One other important observation can be derived from these experiments. No trichomes have been seen on leaf primordia less than 100 μm long (Fig. 2B). For all three genotypes shown in Fig. 7, the initial slope of the plot of trichome number versus leaf length intersects the x-axis at a leaf length of approximately 100 μm, rather than at the origin. This result indicates

Table 4. Average number of trichomes on fully expanded first leaves of all RI lines with recombinations in the *er* m220 interval on chromosome 2

Genotype[a]	Number of trichomes/leaf[b]	Number of leaves examined
CL231 (ER^+ $m220^{Ler}$)	17.4±1.5	10
CL386 (ER^+ $m220^{Ler}$)	10.9±0.4	10
CL35 (*er* $m220^{Col}$)	24.5±1.2	10
CL68 (*er* $m220^{Col}$)	15.2±1.0	10
CL113 (*er* $m220^{Col}$)	18.6±0.9	10
CL209 (*er* $m220^{Col}$)	35.2±1.6	10
CL242 (*er* $m220^{Col}$)	29.1±1.1	10
CL266 (*er* $m220^{Col}$)	15.4±0.9	10
CL283 (*er* $m220^{Col}$)	32.8±1.9	10
CL358 (*er* $m220^{Col}$)	16.9±0.9	10
CL400 (*er* $m220^{Col}$)	16.5±0.6	10

[a]The excess of *er* $m220^{Col}$ recombinants over ER^+ $m220^{Ler}$ recombinants is explained, at least in part, by the two-fold excess of *er* families over ER^+ families among the RI lines (Lister and Dean, 1993).

[b]Mean ± s.e.m. One leaf from the first pair of leaves was examined per plant.

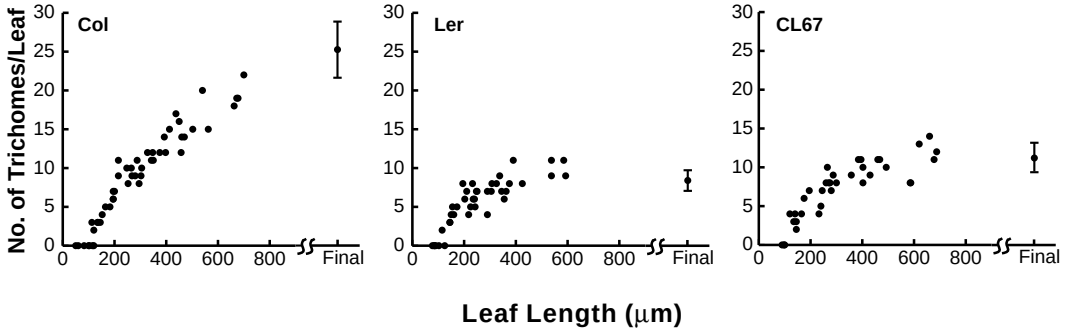

Fig. 7. Comparison of trichome development on first leaf primordia of Col, Ler, and CL67 RI line plants. Final: The mean number of trichomes (± s.d.) on fully expanded leaves of each genotype, after the cessation of trichome development. The data for the other data points represent the values for individual leaf primordia of the indicated lengths.

that trichome initiation commences at a relatively discrete point in leaf development. The present data do not allow us to distinguish between the acquisition of competence or the reception of an inductive signal as the cause of the commencement of trichome development in the protoderm of 100 μm leaves.

DISCUSSION

The results presented here support a model for the initiation of trichome development whereby cells committed to the trichome pathway inhibit their neighbors from developing as trichomes. We have shown that trichomes develop adjacent to one another much less frequently than would be expected by chance alone, and we have presented evidence arguing against an alternative hypothesis that the trichome spacing pattern is achieved by a specialized cell lineage. In previous work, we have shown that upon the commitment to the trichome developmental pathway, the amount of *GL1* transcript increases rapidly from a low basal level (Larkin et al., 1993). This observation suggests that some aspect of the commitment process acts as a positive regulator of *GL1* expression. The *GL1* gene encodes a putative transcriptional activator that is required for trichome development (Oppenheimer et al., 1991). Thus trichome development appears to include both components of the pattern generating mechanism often proposed in theoretical models: a local activator controlled by a positive feedback loop and an inhibitor acting on the surrounding tissue (Turing, 1952; Meinhardt and Gierer, 1974). The nature of the inhibitory signal remains unknown. However, given that the average distance between developing trichomes at the time of their initiation is in the order of three cells (Hülskamp et al., 1994; Larkin et al., 1994), the signal need not function over a distance of more than a few cell diameters. This model of trichome development has many formal similarities to models of the specification of sensory bristles in *Drosophila* (Ghysen et al., 1993; Artavanis-Tsakonas et al., 1995).

The present work also allows greater insight into the timing of trichome development relative to other events occurring in the developing epidermis. Published scanning electron micrographs (Irish and Sussex, 1992; Medford et al., 1992) and our own observations (J. Larkin, unpublished observations) indicate that shortly after the initiation of the first leaf primordium, the adaxial protoderm is approximately five or six cell files wide and only a few cells long. As noted above, the smallest L1 sectors running the full length of the leaf that were detected in our study occupied one-fifth to one-sixth of the width of the primordium. Taken together, these results suggest that the adaxial protoderm consists of five or six cells at the time of leaf primordium initiation. Estimates of the number of adaxial epidermal cells in a mature *Arabidopsis* first leaf range from 10,000 to 18,000 cells (Pyke et al., 1991; Geissler and Sack, personal communication; J. Larkin, unpublished data). Thus cells in the developing epidermis must go through a total of approximately eleven cell division cycles ($6\times2^{11}= 12{,}288$ cells). The first signs of trichome development are not detectable until the leaf primordia have reached a length of at least 100 μm (Figs 2, 7). At this time, the adaxial protoderm consists of approximately 100 cells (J. Larkin, unpublished data), indicating that trichome development begins during the fifth cell cycle. Trichome development starts near the tip of the leaf and proceeds basipetally. In Col plants, the initiation of new trichomes ends in any given region of the leaf at approximately the same time that stomatal mother cells become visible in that region. Stomatal differentiation also proceeds basipetally, starting at the tip of the leaf. Careful in vivo observations of *Pisum* stomatal development by Sachs and coworkers (Sachs, 1978; Kagan et al., 1992) indicate that in any given region of the leaf, stomatal development and the final differentiation of ordinary epidermal cells have the net result of producing approximately a 10-fold increase in the number of epidermal cells (see for example Fig. 2 of Kagan et al., 1992), corresponding to approximately three cell cycles. Although similar developmental studies have not been performed in *Arabidopsis*, anatomical studies of stomatal development in the Brassicaceae (Paliwal, 1967; Pant and Kidwai, 1967) suggest that stomatal development in plants of this family (which includes *Arabidopsis*) involves a similar number of cell divisions. If we assume that stomatal development and epidermal differentiation in *Arabidopsis* account for the last three rounds of cell division, then trichome development must occur during the four cycles that intervene between the time that trichomes begin to form and the onset of stomatal development. During this period, cells committed to develop as trichomes stop dividing and begin to differentiate, while the surrounding protodermal cells continue to divide rapidly. The interaction between the early events of trichome development and the cell cycle remains to be explored.

We have also described a new locus, *RTN*, that affects the number of trichomes produced per leaf, and consequently increases the distance between trichomes on mature leaves. This locus accounts for the majority of the difference in the number of trichomes per leaf between the Col and Ler ecotypes. The RTN^{Ler} allele appears to reduce the length of time that trichomes continue to develop on leaf primordia, in comparison with the Col allele. The protodermal cells continue to divide on Ler leaves, and the onset of stomatal development occurs at approximately the same point as on Col leaves, but no more trichomes are produced. This allele appears to be relatively specific for the Ler background; most other ecotypes that we have examined produce at least as many trichomes per leaf as Col.

One possible explanation for the *RTN* phenotype is that *RTN* encodes an upstream transcriptional activator required to establish the basal expression pattern of *TTG* and/or *GL1*. Alternatively, *RTN* may encode a cofactor that is required for *TTG* or *GL1* function. For either model, the early cessation of trichome initiation on Ler leaves could be explained if the level of RTN product declines during leaf development, and the RTN^{Ler} allele encodes a less active form of the gene product. In this case, the level of RTN activity might fall below the threshold needed for trichome initiation earlier on Ler leaves. Many other models are possible, especially given that only two incompletely dominant alleles of *RTN* are available at present.

The discovery of the *RTN* locus was unexpected, given the exhaustive searches for trichome mutants that have been conducted previously (Koornneef et al., 1982; Hülskamp et al., 1994). Perhaps *RTN* is required for some essential function, rendering strong *rtn* alleles lethal. Also, most screens for chemical- or radiation-induced trichome mutants have been conducted in an Ler background (Koornneef et al., 1982;

Hülskamp et al., 1994), and thus started with the *RTN*^{Ler} allele. In any case, the identification of the *RTN* locus indicates that further genetic and molecular studies will be necessary to identify the complete set of gene products that act in the trichome development pathway.

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