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Arrest of Stomatal Initials in *Tradescantia* Is Linked to the Proximity of Neighboring Stomata and Results in the Arrested Initials Acquiring Properties of Epidermal Cells

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We examined spatial relations of arrested stomatal initials and their differentiated state on leaves of the monocotyledon *Tradescantia*. The placement and proximity of stomata and arrested stomatal initials to the five nearest stomata were studied to test the hypothesis that if developing stomatal initials occur too close to one another, initials will arrest. The results showed that arrested stomatal initials were not randomly placed, but were closely associated with another stoma, most often in an adjacent cell file. The distance to their nearest stomatal neighbors was less than the equivalent distance between stomata that mature. After stomatal initials form, their position within or across cell files was not adjusted by cell division or expansion. Synergistic effects from several neighboring stomata could not be linked to stomatal arrest; rather, arrest was associated only with the nearest stomatal neighbor. Since the arrest of stomatal initials was distance dependent, a failure intrinsic to the arrested initials is not solely responsible for halting stomatal development. These data show that an inhibitory mechanism adjusts stomatal development to influence the final distribution of *Tradescantia* stomata. The pigmentation and expansion characteristics of arrested stomatal initials were like those of epidermal cells, indicating that the initials did not remain halted at a specific point in their development. The capacity of arrested initials to differentiate in the epidermal cell pathway indicates that they remain pluripotent after their initial specification and that the opportunity for patterning is long enough to permit their entry into the epidermal cell pathway. © 1995 Academic Press, Inc.

INTRODUCTION

The arrest of stomatal initials or the abortion of developing stomata commonly occurs in leaves of monocotyledonous and dicotyledonous species and indicates that stomata are not determined at their origin. The cause of arrest, the location of arrested initials, and the differentiated state of mature arrested initials are not known. Our objective was to study these arrested stomatal initials in *Tradescantia* because they can provide information on the patterning process, the determination of stomata, and cellular specification as it occurs in the leaf epidermis.

In studying these arrested initials, two developmental questions were put forward. The first question was what is the location of the arrested initials with respect to nearby stomata. We reasoned that if the location of arrested stomatal initials were random, then the interruption in their development is not influenced by external factors, but is solely intrinsic. Alternatively, if the position of arrested stomatal initials were predictable and regular, then external factors influence their arrest and affect the final distribution of stomata.

The second question was what is the differentiated state of mature arrested initials. If some initials arrest because too many form near one another, then they likely undergo a change in development. Differentiation might proceed in one of these ways: (1) the initial might not divide to form guard cells, but exhibit their cellular characteristics, (2) the initial could remain halted in development, but expand to keep pace with the growing epidermal tissue, or (3) the initial could switch to another pathway. The fate of arrested initials is of interest because the temporal window of patterning and a cell's capacity for continued development can be examined. Although plant cells are well known for their totipotency and for their ability to dedifferentiate under extraordinary conditions, what happens in this common instance when a cell's development is halted early in the pathway? What becomes of a cell that was originally destined to the stomatal pathway, but arrests before completing its development? Is it capable of additional development, can it acquire characteristics of another cell type, or does it simply become a space holder?

We used leaves from the monocot *Tradescantia* to answer these questions. Monocot leaves are well suited for such a study because growth occurs from a basal meristem that generates new cells in regular, longitudinal files toward the leaf tip. As cells are displaced from the

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meristem in immature leaves, they proceed to differentiate into either epidermal cells or stomata. Each cell file thus serves as a profile of development, from less advanced to more advanced developmental stages, when viewed from leaf base to tip. This mode of growth contrasts with that of dicot leaves, in which cell division is dispersed in a mosaic pattern throughout the leaf and stomatal ontogeny is more difficult to trace.

The results showed that the spatial distribution of arrested stomatal initials depends on the distance to the nearest neighboring stomata. Thus, we concluded that a failure intrinsic to the arrested initials is not solely responsible for halting stomatal development, but that an inhibitory mechanism contributes to the final distribution of *Tradescantia* stomata. Since mature arrested initials acquired traits and growth characteristics of epidermal cells, the time interval for specifying cells is long enough to permit the arrested stomatal initials to become epidermal cells.

MATERIALS AND METHODS

Source of data. Clonal material of green and anthocyanin-accumulating varieties of *Tradescantia* sp. were propagated and grown as previously described (Croxdale *et al.*, 1992).

Identifying arrested stomatal initials. Arrested stomatal initials were recognizable throughout stomatal development by their tabular shape in face view, the absence of subsidiary cells, and their smaller size compared to surrounding epidermal cells (Figs. 1A and B). Stomatal development proceeds sequentially within cell files, with stomata at more advanced stages of development toward the leaf tip (Croxdale *et al.*, 1992). The arrest of stomatal initials could be diagnosed at all stages of stomatal development where subsidiary cells were present; arrested initials were evident when stomatal initials toward the base in the same cell file had subsidiary cells (Fig. 1A).

Distance and position measurements. A stomatal complex or an arrested stomatal initial was chosen at random as a reference cell, and the distances from that cell to its five nearest stomata were recorded (Fig. 2). The five distances for each observation were ranked 1 through 5, with rank 1 representing the nearest neighbor and rank 5 representing the fifth nearest neighbor. In immature leaves, the positions of the nearest five stomatal complexes were recorded in addition to the distance measurements.

The position of each stomal neighbor was evaluated by its cell file (same, adjacent, or peripheral) and by its position within the file, relative to the reference cell. Figure 4 depicts three sets of such positional data. The location of the stomatal neighbors are given by cell file and by cell in the file, based on the reference cell at the center. The numbers in a given cell refer to the number of times stomatal neighbors were found at this location for the reference cell indicated. Measurements were made on 60 reference cells of each type (arrested initial, stoma) for immature leaves and on 80 reference cells of each type for mature leaves.

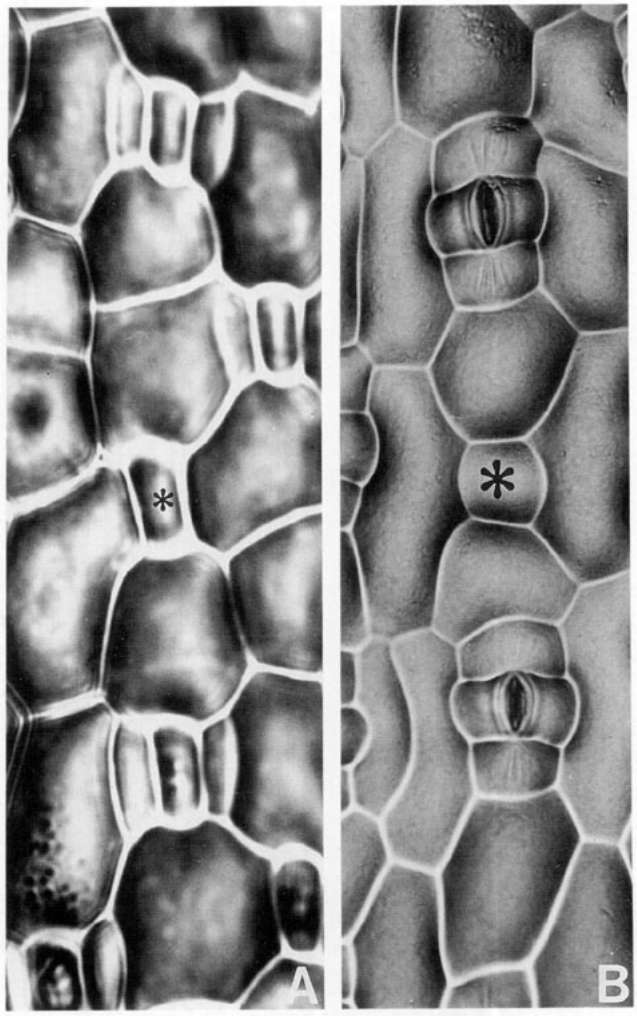

FIG. 1. (A) An arrested stomatal initial (asterisk) in the immature leaf region located between stomatal initials with their first pair of subsidiary cells. ×400. (B) An arrested stomatal initial (asterisk) found in the mature leaf. ×100.

To explore spatial relationships within cell files, the positions of stomatal complexes and arrested stomatal initials were recorded at differentiating, expanding, and mature stages of stomatal development. The position of a reference cell, chosen at random as above, was assessed relative to the nearest two stomata in the same cell file, i.e., toward the leaf base and toward the leaf tip. This was done by counting the number of intervening epidermal cells and by measuring the distance from the

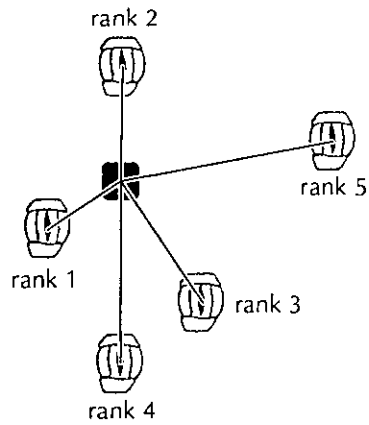

FIG. 2. A diagram representing one possible configuration of the five nearest stomatal neighbors around a reference cell (stoma or arrested stomatal initial, shown in black). Neighbors were ranked from one to five based on their proximity to the reference cell.

reference cell to the nearest neighboring stomata in the cell file.

The epidermis of the immature leaves studied here contained two regions: one of differentiating stomatal initials in which divisions that form stomatal complexes were still taking place and one of stomata that had completed cell division but had not fully expanded. The epidermis of mature leaves had fully expanded, differentiated stomata. The immature leaves selected were between 11 and 17 mm in length (10 leaves, average 14 mm) and mature leaves were between 39 and 50 mm in length (17 leaves, average 45 mm). Leaf replicas were made by pressing the lower surfaces of leaves onto slides coated with cyanoacrylate glue and peeling away the leaves after the glue dried. All measurements were taken directly from leaf impressions on microscope slides using a calibrated image analysis system (Cue 2, Olympus Corporation) interfaced with a compound microscope. Distance measurements were recorded from the centers of cells in face view.

Cell measurements. We recorded the length (parallel to the cell file) and width of newly formed stomatal initials, mature arrested initials, and mature pairs of guard cells in face view. Additionally, depth measurements were taken on mature arrested initials and guard cell pairs using leaf transections. Since newly formed stomatal initials and epidermal cells can not be distinguished from one another in transection, measurements of undifferentiated epidermal cells were taken in the basal 2 mm of the immature leaves where stomatal differentiation is occurring (Chin *et al.*, 1994, companion paper). The depth of mature stomatal complexes and epidermal cells was measured from transections in the central regions of mature leaves.

We used mature leaves ranging from 47 to 60 mm in length and immature leaves approximately 5 mm in length. Measurement were collected from 170 samples on 50 mature leaves and from 105 samples on 25 immature leaves. Measurements were taken from fresh tissue, cyanoacrylate glue replicas of the abaxial leaf surface, and SEM micrographs of young leaves or their impressions with the assistance of the image analysis system described above.

Microscopy. Specimens were examined in surface and transectional (50 μm sections) views with a Zeiss Axioplan microscope using bright-field and fluorescence optics (filter pack 395-400/FT 460/LP 470). Specimens were photographed with Kodak 2415 or Kodacolor 400 film.

RESULTS

Distance relationships of arrested initials and stomata. The five nearest stomata of each type of reference cell, stoma and arrested initial, were ranked by their distance, with rank 1 representing the nearest neighbor and rank 5 representing the fifth nearest. The distances for arrested initials were significantly shorter than those for stomata for all five ranks in both immature and mature leaves (Student's t test, $P < 0.01$ in all cases; Fig. 3).

There was an unexpected relationship between the average distances for ranked neighbors of arrested initials and those of stomata. The distances for the rank 2 through 5 neighbors of arrested initials closely resembled the distances for the rank 1 through 4 neighbors of stomata (Fig. 3). This relationship held for both immature and mature leaves.

Distance to neighboring stomata analyzed by cell file. In immature leaves, rank 1 distances for stomata and arrested initials were evaluated by cell file as follows: (1) neighbors found in the same cell file as the reference cell, (2) neighbors in a cell file adjacent to that containing the reference cell, and (3) neighbors to the periphery of the adjacent cell files (Table 1). Distances corresponding to neighbors in adjacent cell files were significantly shorter for arrested initials (24.8 ± 4.4 μm) than for stomata (30.9 ± 5.6 μm; independent t test, $P < 0.001$). For neighbors located in the same cell file as the reference cell, distances were still shorter for arrested initials than stomata (33.8 ± 7.2 μm compared to 38.5 ± 4.8 μm); however, the difference was much less significant ($P = 0.039$ for pooled variances, $P = 0.084$ for separate variances). Arrested initial neighbors in adjacent files were 26.6% closer on average than neighbors in the same file, while stomatal neighbors in adjacent files were 19.6% closer than neighbors in the same file. For arrested initials, distances to rank 1 neighbors in peripheral files varied widely, but were still shorter on the whole than for sto-

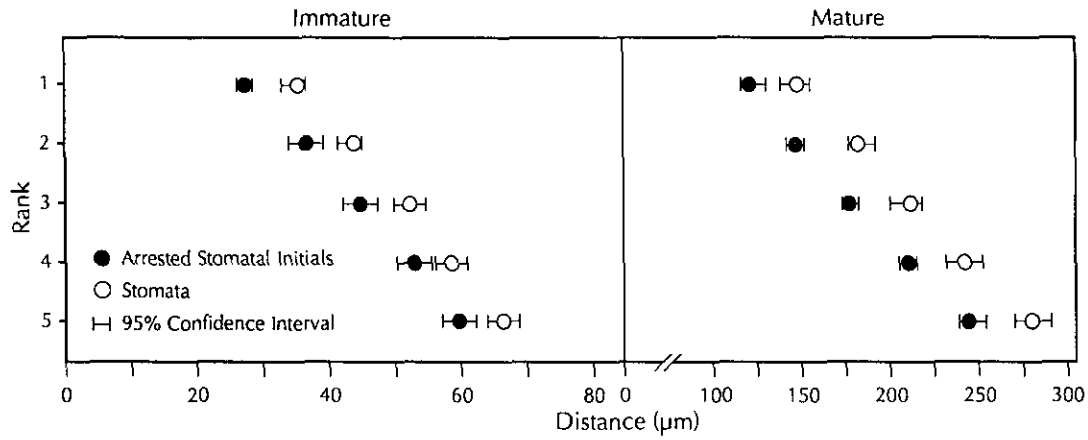

FIG. 3. Average distances (μm) from reference cells (arrested stomatal initials ● and stomata ○) to neighboring stomata (ranked 1 through 5, with rank 1 the nearest neighbor). Each rank is shown separately. $N = 60$ for each cell type (stomata and arrested stomatal initials) in immature leaves, and $N = 80$ for each cell type in mature leaves. Horizontal bars associated with each data point indicate 95% confidence intervals.

mata (31.3 ± 11.4 μm versus 39.5 ± 4.7 μm; $P = 0.033$ for pooled variances, $P = 0.045$ for separate variances).

The occurrence of stomatal neighbors by cell file. In immature leaves, the occurrence of the five nearest neighbors of stomata and arrested initials was evaluated by cell file (the same, adjacent, or peripheral file relative to the reference cell; see Fig. 4 for a schematic display of selected data). Two different χ^2 tests were used to analyze the file incidence for the five ranks (Table 2). One used incidence values of arrested initial neighbors as observed values and incidence values of stomatal neighbors as expected values. The other treated incidence values for stomata and arrested initials as observed values subject to variation. Analysis showed that only the file incidence for the rank 1 neighbors was reliably different for arrested stomatal initials and stomata. The same result was found for both χ^2 tests (95% confidence level; Table 2).

For arrested initials, the occurrence of rank 1 neighbors in an adjacent cell file was four times greater than their occurrence in the same cell file (39 compared to 10; Table 2). In contrast, the occurrence of rank 1 neighbors of stomata was only slightly greater in adjacent cell files than in the same file (27 compared to 21). The incidence of rank 1 neighbors in peripheral cell files was similar for both types of cells (11 for arrested initials, 12 for stomata).

The nearest neighbor of an arrested initial was most commonly found in an adjacent cell file and in the closest lateral locations (Fig. 4). The rank 1 neighbors of stomata showed no such tendency (Fig. 4). The distribution of the rank 2 neighbors of arrested initials showed no cell file or location preference.

The placement of arrested initials and stomata within a file. The position of arrested stomatal initials was studied to determine if these cells had a consistent placement within a cell file that might indicate the source of their arrest, i.e., that a stoma of the same file was involved in inhibiting development. This was assessed by measuring the distances from the arrested initial to the nearest stoma in each direction. Within cell files, the distance between the arrested initial and the nearest stoma was not predictable, i.e., not regularly closer to the tip or to the basal stoma (Fig. 5). The variation in distance relationships was similar at the three observed stages of stomatal development (differentiating, expanding, mature; Fig. 5).

The number of cells separating mature arrested initials from the nearest stomatal neighbors was not statistically different from the number separating stomata (χ^2 test, 95% confidence level). For arrested initials, 52% had one intervening epidermal cell to the nearest neighboring stoma, 23% had two epidermal cells, 12% had three epidermal cells, and 13% had four or more cells.

TABLE 1

DISTANCE (μm) FROM ARRESTED STOMATAL INITIALS (ASI) AND STOMATA (S) TO NEAREST NEIGHBORING STOMA IN IMMATURE LEAVES BY CELL FILE

	ASI		S	
Cell file	*Distance* (μm) SD	*N*	*Distance* (μm) SD	*N*
Adjacent[a]	24.8 ± 4.4	39	30.9 ± 5.6	27
Same	33.8 ± 7.2	10	38.5 ± 4.8	21
Peripheral	31.3 ± 11.4	11	39.5 ± 4.7	12

[a] Significantly different (independent *t* test, $P = <0.001$).

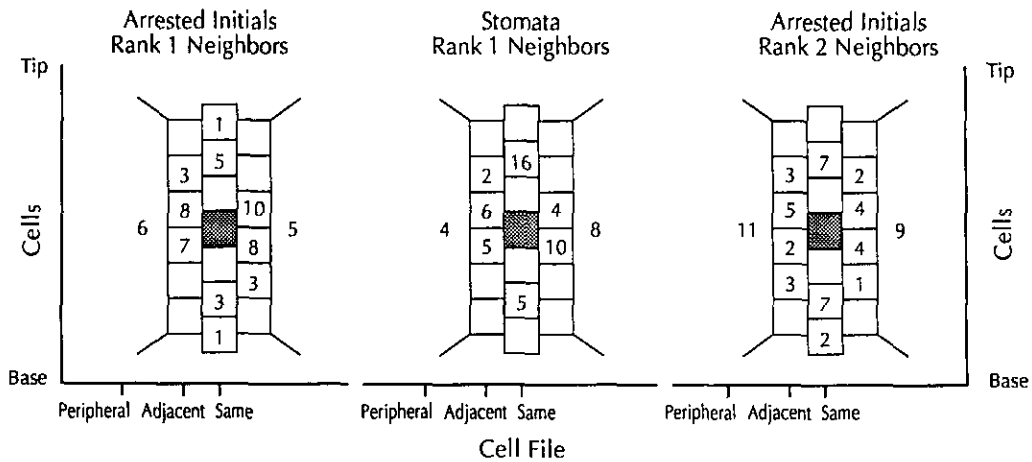

FIG. 4. Schematic tissue maps showing the positional incidence of rank 1 neighbors for arrested initials and stomata and rank 2 neighbors of arrested initials in immature leaves. The reference cell (shaded, arrested initial or stoma) is in the center of each map. Numbers in cells refer to the incidence of the neighboring stomata in those cell positions. $N = 60$ for each cell type.

For stomata, these percentages were 50, 30, 10, and 10%, respectively.

Examining the possibility of synergism. Variances of the ranked neighbor distances (Fig. 3) were calculated to examine whether arrest was a combined effect from several stomatal neighbors. Synergism would be apparent if ranked neighbors of arrested initials had distances with lower variances than those for equivalent ranks of stomata. We found no appreciable differences between variances for arrested stomatal initial and stomatal distances. In another test of synergism, the distances of rank 1 neighbors were plotted against those of the rank 2 neighbors. No association between neighbors of arrested initials or stomata was apparent in either the immature or mature leaves.

TABLE 2

INCIDENCE BY CELL FILE FOR THE FIVE NEAREST NEIGHBORING STOMATA OF ARRESTED STOMATAL INITIALS (ASI) AND OF STOMATA (S) IN IMMATURE LEAVES[a]

	Same cell file		Adjacent cell file		Peripheral cell file	
Rank	ASI	S	ASI	S	ASI	S
1[b]	10	21	39	27	11	12
2[c]	16	23	24	12	20	25
3	11	13	16	10	33	37
4[c]	15	15	15	6	38	45
5	12	8	10	7	38	45

[a] $n = 60$ for each rank of neighbors for arrested stomatal initials (ASI) and stomata (S), where rank 1 represents the nearest stomata and rank 5 represents the most distant stomata.

[b] For rank 1, cell file incidence for neighbors of arrested stomatal initials (ASI) is significantly different than incidence for stomata (S) at greater than 95% confidence according to both the equation in[c] and: $\chi^2 = \Sigma(e - o)^2/(e + o)$ ($df = 2$), where e = incidence for neighbors of stomata and o = incidence for neighbors of arrested initials.

[c] Incidence for neighbors of arrested stomatal initials (ASI) is different from that for neighbors of stomata (S) for ranks 1, 2, and 4 at greater than 95% confidence according to: $\chi^2 = \Sigma(e - o)^2/e$ ($df = 2$).

Pigmentation in mature epidermal cells. In the purple variety of *Tradescantia*, arrested initials (Fig. 6, arrowhead) and epidermal cells accumulated anthocyanin, but cells associated with the stomatal complexes did not (Fig. 6). Anthocyanin concentration appeared to be greater within the arrested initials than in the epidermal cells. Cells in the stomatal complex appear green in the figure as a result of light passing through the chlorophyll-enriched mesophyll region below the epidermis (Fig. 6). If the stomatal complex were viewed in isolation, the two central guard cells would contain chloroplasts and the subsidiary cells would be colorless.

Transectional appearance of mature arrested initials. Based on their dimensions in surface view (data presented below), arrested stomatal initials in transection could be distinguished at maturity from epidermal cells and cells of stomatal complexes. At maturity, arrested initials had a wedge-shaped appearance in profile, with the tip of the wedge extending nearly to the mesophyll layer as epidermal cells do (Fig. 7B). Although stomatal complexes always appeared above large substomatal cavities (Fig. 7A), arrested initials lack such cavities (Fig. 7B).

Expansion of epidermal cells. In face view, young stomatal initials were nearly square, with a width of 8.3 ± 1.7 μm and a length of 7.8 ± 1.2 μm (Table 3). Those initials that arrested retained their basic shape at maturity (62.2 ± 9.2 μm in width, 58,2 ± 7.4 μm in length). However, initials that divided and completed their development had guard cell pairs that were significantly

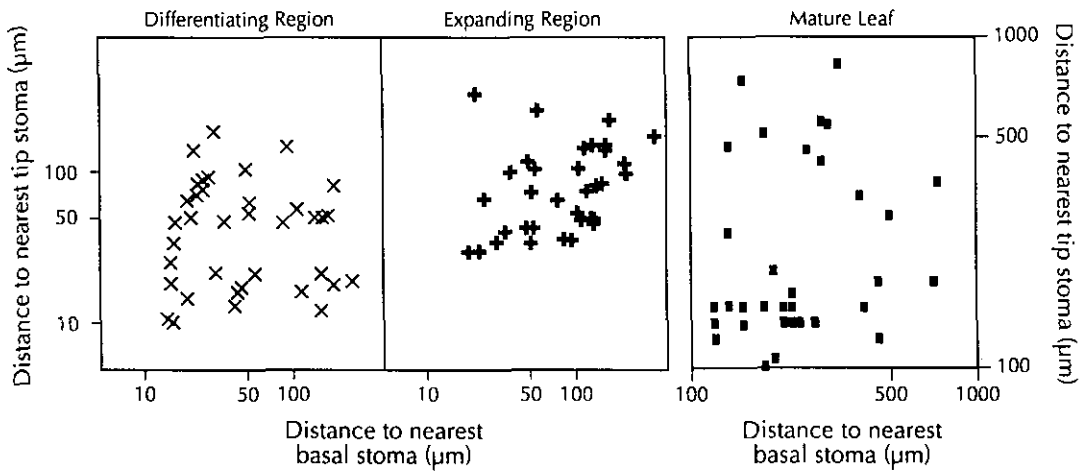

FIG. 5. Distance relationships between arrested initials and the nearest stomata within the same file in developing leaves at three different stages of development. Scatterplots show the position of each arrested initial as a function of distance to its nearest neighbors toward the leaf tip and toward the leaf base.

longer (47.1 ± 3.8 μm) than they were wide (30.7 ± 4.3 μm, Table 3). When we compared the length and width of arrested initials with those of young stomatal initials (Table 3), we found that the arrested initial expanded 7.5 times in both dimensions. Stomatal initials that completed their development as guard cell pairs expanded much less, approximately 4 times in width and 6 times in length (Table 3).

The average depth of the immature abaxial epidermis was 10.7 ± 0.6 μm, while the mature epidermis was 91.4 ± 13.2 μm in depth (Table 3). The average thickness of a mature arrested initial was 75.8 ± 10.4 μm and of a guard cell was 17.4 ± 4.9 μm (Table 3). The epidermal cells expanded nearly 8.5 times in thickness during development, while the arrested initials expanded about 7 times in the same dimension (Table 3). In contrast, the mature guard cells pair was less than twice the thickness of an undifferentiated epidermal cell (Table 3).

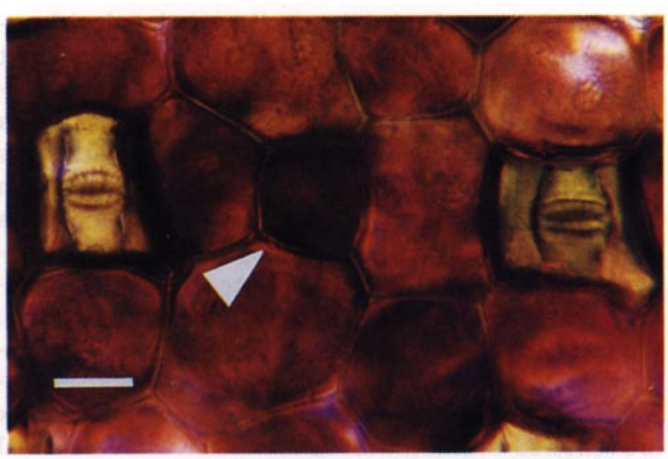

FIG. 6. The epidermis of the anthocyanin-accumulating variety of *Tradescantia* epidermis in face view showing stomatal complexes, epidermal cells, and a mature arrested initial (arrowhead). The red pigment located in the epidermal cells and arrested stomatal initial is anthocyanin. Cells of the stomatal complexes appear green as a result of light passing through the chloroplasts in the mesophyll cells below the epidermis. Bar, 28 μm.

DISCUSSION

Bünning suggested that the origin of new stomata is regulated by inhibition, i.e., that a given distance must be present between existing stomata before a new stoma will arise (Bünning, 1956). He put forward this theory to explain stomatal origin in dicotyledonous species, but without addressing the complication that the mosaic growth of the leaves would have on this theory. If the inhibitor mechanism operated perfectly, then arrested or aborted stomata would never result and all stomata would complete their development. However, arrested stomata commonly occur in both monocotyledons and dicotyledons.

In *Tradescantia* leaves, nearly 10% of the stomatal initials do not complete their development, but appear as large arrested initials in mature leaves (Croxdale *et al.*, 1992). Since the frequency of arrested initials on a per cell basis is the same in immature and mature leaves, arrested initials are not merely individual stomatal initials that are slow to develop. In fact, stomatal differentiation takes place in a given zone of the leaf and the development of arrested stomatal initials is invariably interrupted at the stomatal initial stage (Croxdale *et al.*, 1992).

Stomatal neighbors of arrested stomatal initials. We found the distance from an arrested stomatal initial to its nearest stoma to be shorter than the equivalent dis-

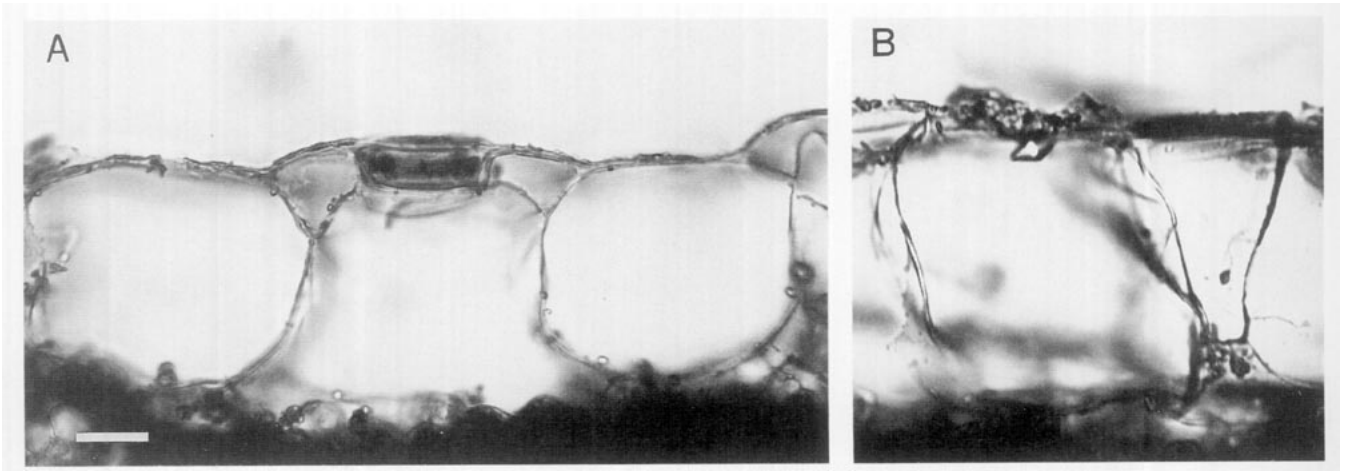

FIG. 7. (A) Micrograph of a mature stomatal complex in transection. The complex is bounded on the left and right by large epidermal cells that extend to the mesophyll region. Directly beneath the stomatal complex is an empty space termed the substomatal cavity. Bar, 18 μm. (B) Micrograph of a mature arrested initial, a truncated cone, in transection. The arrested initial has expanded and nearly reached the mesophyll layer. Magnification as in A.

tance from a mature stoma. The distance-dependent relationship indicates that developmental arrest of stomatal initials did not result solely from an intrinsic failure of the initial.

When analyzing spatial relationships of neighboring stomata in the same cell file, neither their distance nor predictable position (closer to the leaf base or tip) correlated with the arrest of an initial. Rather, the observed distance dependence was found when considering neighboring stomata in adjacent cell files. *Tradescantia* stomatal differentiation produces a minimum spacing of one epidermal cell between stomatal initials in the same cell file (Croxdale *et al.*, 1992). However, between cell files, cells are staggered and there is no such constraint. As a result, it is possible for two stomatal initials in adjacent cell files to be physically closer to one another than initials in the same cell file. Thus, the nearest stoma of an arrested initial was most often, although not always, found in an adjacent cell file. Although it might seem that arrest is also the result of positional dependency, distance is the critical component in arrest. The common position of the nearest neighbor results from the cell geometry of the epidermis.

TABLE 3
MEAN CELL DIMENSIONS μm (± SD) AND EXPANSION CHARACTERISTICS IN THE *Tradescantia* LEAF EPIDERMIS

		Leaf age		
Dimension	Cell type	Immature (SD)[a]	Mature (SD)	Expansion ratio[b]
Thickness	Arrested initial	10.7 (0.6)	75.8 (10.4)	7.08[b]
	Guard cell pair	10.7 (0.6)	17.4 (4.9)	1.62
	Epidermal cell	10.7 (0.6)	91.4 (13.2)	8.54
Length	Arrested initial	7.8 (1.2)	58.2 (7.4)	7.46
	Guard cell pair	7.8 (1.2)	47.1 (3.8)	6.03
Width	Arrested initial	8.3 (1.7)	62.2 (9.2)	7.49
	Guard cell pair	8.3 (1.7)	30.7 (4.3)	3.69

[a] The values are the same for all cell types in immature leaf regions because differentiation has not taken place.

[b] The expansion ratio is the value of the dimension at maturity divided by its value at the immature stage.

The proximity of stomatal initials in adjacent cell files and the absence of subsidiary cells associated with arrested stomatal initials might lead one to suspect that the distance data for arresting stomatal initials is artifactual, i.e., that initials are closer to the nearest stoma because they lack subsidiary cells. We considered this possibility and discounted it for two reasons. First, in immature leaf regions the arrested stomatal initial is centered in the file and is the same width as are developing stomatal initials (Fig. 1A). Since the distance measurements were made from the center of the initial cells, the presence or absence of subsidiary cells does not influence the measurements. At maturity, the same relationship holds, the guard cells of the stomatal complex are centered within the file as are the arrested stomatal initials (Fig. 1B). Second, we evaluated ratios of the distance from arrested and developing stomatal initials to their nearest neighbors in adjacent and in the same cell files. The ratios show that the nearest neighbors in adjacent files were closer to arrested initials (27%) than to developing stomata (19%). Hence, the interpretation of

the distance data is accurate; the absence of subsidiary cells does not censor the distance data.

Agreement between average distances of the ranked stomatal neighbors in immature and mature leaves indicated that tissue expansion was proportional and did not modify the original stomatal relationships. The number of intervening epidermal cells and relative distance of arrested initials to their nearest neighbors within cell files also did not change during development. Therefore, we concluded that neither cell division nor tissue expansion adjusted the placement of arrested initials. The distance relationships established upon the origin of stomatal initials in the immature region of the leaf were maintained in the mature regions of the leaf.

We attempted to follow arrest of stomatal initials *in vivo* by making replicas of the immature leaf surface over time. Although dental impression material has been used frequently with plant tissues (Williams and Green, 1988), we have been unsuccessfull in making replicas of small *Tradescantia* leaves. Leaves of interest, which *in situ* are enclosed by the sheaths of older leaves, range in size from 2 to 20 mm in length (Chin *et al.*, 1994, companion paper). They have little cuticle to protect them from desiccation. When replicas have been attempted, the leaves shrivel before the impressions form and then die, usually within a few hours.

The variances between ranked distances were analyzed to pursue evidence for a combined effect of two or more neighboring stomatal initials on arrest. None of the arrested initial variances indicated paired or collective associations between two or more neighbor ranks. In fact, variances among the ranks of neighbors for both cell types were not different. In another test of synergism, the distances of the rank 1 and rank 2 neighbors for arrested initials showed no correlation to one another. The average distances of rank 2 through 5 neighbors of arrested initials were similar to those of rank 1 through 4 neighbors of stomata. If more than one neighbor were implicated in arrest, the neighbor ranks involved would deviate rather than parallel the ranks of the stomatal neighbor array. Based on this evidence, we conclude that arrest was linked only with the nearest stoma.

Our results in *Tradescantia* established that developmental arrest results from a distance-dependent relationship of stomatal neighbors. This distance dependency evokes inhibition models in which stomatal spacing has a minimum distance requirement. Such models typically refer to an inhibitor that prevents the formation of new initials within the threshold range of the inhibitor (Bünning, 1956; Korn, 1981; Leick, 1954; Meinhardt, 1982). In *Tradescantia* this influence would halt the development of existing stomatal initials and not allow the formation of subsidiary cells to take place. Inhibition may result from a passive rather than an active influence. In such a model, competition for a limited resource (a nutrient, metabolite, or hormone) might permit the continued development of one stomatal initial at the expense of an immediate neighbor. Regardless of the arresting mechanism, a threshold distance for these models may be deduced from the minimum distance of initials that successfully develop into stomata, e.g., the distances of the rank 1 neighbors.

Arrested stomatal initials and the patterning process in monocotyledons. Bünning asserted that stomatal patterning in monocotyledons was determined by the pattern of cell division and in dicotyledons resulted from inhibition by existing stomata. The arrested initial data presented for *Tradescantia* clearly shows that inhibition was involved in the continuation of stomatal development. Sachs (1974) interpreted the pattern of stomatal distribution in the monocot *Crinum* as the result of cell division, a determinative process, rather than the result of a regulatory process involving influences and interaction between developing stomata. The conclusion was based on distance measurements between mature stomata, but excluded arrested stomatal initials that were present. By not measuring distances from the arrested stomatal initials to mature stomata in *Crinum*, it was not possible to ascertain whether the position of mature stomata was correlated with the cessation of the arrested initials. While *Crinum* stomata might arrest on a random basis, measurements of their distance to neighboring stomata would have to be made to evaluate this aspect. *Crinum* initials also may arrest based on distance to neighboring stomata as they do in *Tradescantia*.

The location of arrested stomatal initials seldom has been studied in monocotyledons. However, the separation of monocot stomata from one another has been reported as being at least one epidermal cell in the polar and lateral positions (Stebbins and Jain, 1960; Sachs, 1974; Rassmussen, 1986). In *Sansevieria* and *Ruscus*, many stomata that originate also do not complete their development (Kagan and Sachs, 1991; Sachs *et al.*, 1993). In these species, stomatal distribution is more ordered at maturity than at the onset, although whether the difference in order is significant was not assessed (Kagan and Sachs, 1991; Sachs *et al.*, 1993). If the difference in their order is significant, then arrest must occur in a distance-dependent fashion. The adjustment in stomatal distribution was attributed to epigenetic selection, i.e., certain stomata are selected for continued development from a large population of developing stomata. Although a mechanism of selection was not put forward, epigenetic selection has been interpreted as a variant of the inhibition model (see Held, 1992).

Charlton's theory of stomatal patterning (1990) hypothesizes that cells are patterned into the stomatal

pathway based on their cell cycle position as they are displaced through the patterning region of the leaf. He also suggested that aborted stomata (arrested stomatal initials) might result based on their cell cycle position. That is, after cells are patterned to become guard mother cells (stomatal initials), some mother cells would not divide based on their cell cycle position upon reaching the leaf zone where guard cells form. Given that *Tradescantia* stomatal initials arrest with distance dependency, the explanation is unlikely in this species. To test this hypothesis it would be necessary to know the cell cycle dynamics of the epidermal cell population and superimpose this information on cells as they progress or arrest in the stomatal development pathway. Charlton's cell cycle theory may be valid for the initial patterning of cells to the stomatal pathway, but it is an improbable explanation of stomatal arrest in *Tradescantia*.

The distance dependency of the arresting stomatal initial in *Tradescantia* fits a threshold model of inhibition, a common means of regulating specialized cell types in biological organisms. Examples of such regulation are known in heterocysts of the filamentous cyanobacterium *Anabaena* (Wilcox *et al.*, 1973), in the spatial organization of epidermal structures (Held, 1991; Orenic *et al.*, 1993), and the ommatidia of fruit flies (Rubin, 1989). Activators (Wigglesworth, 1940) and activators plus inhibitors (Richelle and Ghysen, 1979; Simpson, 1990) have been suggested as determinants of *Rhodinus* and *Drosophila* bristle pattern, although the identity of the molecules is not yet known.

Differentiation in mature arrested stomatal initials. It is well known that plant cells have the capacity to develop first as one cell type and then dedifferentiate and become another cell type. This occurs in interfascicular cells (Warren Wilson and Warren Wilson, 1984) and in epidermal cells of carpels (Walker, 1975; Verbeke, 1992). The capacity of a cell to dedifferentiate is different from the situation studied here. *Tradescantia* stomatal initials change before their differentiation is complete and, therefore, do not dedifferentiate. Plant cells can be induced to change their development, e.g., isolated fern leaf primordia can become shoots (Cutter, 1954) and pollen grains can generate haploid embryos (Guha and Maheshwari, 1964). However, these switches do not occur spontaneously, but only following drastic changes in a cell's environment. These switches reflect the totipotency of plant cells subjected to dire circumstances rather than the change to another cell type which is a routine occurrence of differentiating epidermal tissue.

Although initially directed to stomatal development, arrested initial cells at maturity share characteristics with epidermal cells. These characteristics include: the presence of anthocyanin, their expansion toward the leaf interior, and their lack of a substomatal cavity. Arrested initials of *Tradescantia* also do not divide to form guard cells (Croxdale *et al.*, 1992). These traits of mature arrested initials indicate that although their progress in the stomatal pathway was halted, their development did not cease. They acquired characteristics of epidermal cells, revealing that they remained pluripotent after their formation and that the window of patterning is long enough for them to become another cell type of the epidermis.

In the anthocyanin-accumulating variety of *Tradescantia*, we found that mature arrested initials contained the pigment. No cells of the stomatal complex contained anthocyanin, only the epidermal cells. Arrested stomatal initials acquired a secondary metabolite of epidermal cells. Since this pigment is also not present in undifferentiated cells of the epidermis (Croxdale, data not presented), mature epidermal cells exhibit differentiation that must result from active processes. While it might be tempting to consider that arrested stomatal initials merely default to the epidermal cell pathway, the evidence indicates that this is not true. There must be a mechanism that regulates the presence of the pigment in particular cell types. The mechanism may reside either in the epidermal cells themselves or a signaling system may direct the compound to epidermal cells. Although every cell in the epidermal tissue is, by definition, a kind of epidermal cell, epidermal cells themselves differentiate and are recognizably different from the trichomes or stomatal complexes of the epidermal tissue. Use of molecular probes specific for epidermal cells would be a useful demonstration of their differentiation (Clark *et al.*, 1992).

When the expansion of epidermal cells and stomata in face and profile views was compared, the growth of arrested stomatal initials also indicated that they switched from the stomatal pathway to the epidermal cell pathway. Guard cells and subsidiary cells of the stoma expanded little toward the mesophyll layer, while the surrounding epidermal cells extended to cells of the mesophyll. The difference in expansion between stomatal and epidermal cells resulted in the passive formation of the substomatal cavity beneath the stoma. In contrast, the mature arrested initials expanded toward, and nearly reached, the mesophyll layer as did the epidermal cells. Comparing the dimensions of mature arrested initials in face view with those of guard cell pairs showed that expansion of arrested initials was unlike that of initials that complete their development. Arrested initials occupied nearly the full width of a cell file, in contrast to the four cells, two guard cells, and two subsidiary cells that fill this space when a stomatal complex forms. Regardless, the similar expansion characteristics of arrested stomatal initials and epidermal cells make it

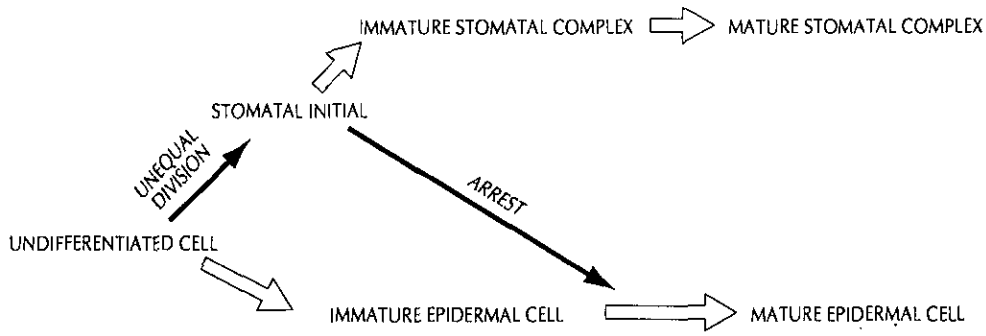

FIG. 8. Developmental pathways of young, undifferentiated cells of the *Tradescantia* epidermis. Nearly 20% of the undifferentiated cells divide unequally and produce stomatal initials. Most of the initials, 90%, complete their development and form stomatal complexes. However, the remaining initials arrest and acquire characteristics of epidermal cells. Larger arrows represent a larger proportion of cells proceeding in a particular pathway.

clear that the development of the arrested initial is closely related to that of the epidermal cells. Arrested stomatal initials switch pathways.

A similar pathway switch is known in the heterocysts of the prokaryote *Anabaena* (Wilcox *et al.*, 1973). Those specialized cells fix nitrogen and arise from proheterocysts. When proheterocysts are too close to each other, one of them reverts to the nonheterocyst cell type, while the other continues its development. The cell that changes state becomes photosynthetic. The causative agent of the change to the unspecialized condition is unknown, although glutamine or one of its derivatives is likely (Buikema and Hazelkorn, 1991; Wolk, 1979); other compounds also might be candidates for initiating change (Wolk, 1991). The demonstration that both prokaryotic and eukaryotic cells switch developmental pathways indicates its fundamental importance in development.

These changes in cell fate bring to mind a Darwinian mechanism of patterning (Edelman, 1987; Held, 1992). The basic premise of this mechanism is that more cells are specified to a particular fate than will eventually complete their development. Those cells that will not complete their development die or change their state. Although animal cells have the capacity to migrate to areas appropriate for their development (Yoshida and Aoki, 1989), this is not an option for plant cells. The death of animal cells or their change in state has been suggested to occur by a random process (Held, 1992), but in *Tradescantia* the arrested initials are selected based on distance to neighboring initials. Pathway switches may be one mechanism to accomplish Darwinian patterning.

Model of patterning for undifferentiated epidermal cells. We suggest the following model for the patterning of undifferentiated cells in the epidermis of *Tradescantia* leaves (Fig. 8). Patterning occurs after all proliferative divisions in the epidermis are completed. Stomatal patterning is evident when undifferentiated cells, approximately 20%, undergo an unequal division producing a small stomatal initial and a large sister cell (Croxdale *et al.*, 1992). The remaining undifferentiated cells become epidermal cells at maturity; the timing of their determination and differentiation is not established. Stomatal initials are determined by the time the first pair of subsidiary cells appears with the initial. A majority of stomatal initials form mature complexes; however, their continued development is contingent upon their position exceeding a minimum distance, 30 μm, from the nearest neighboring initial. If they occur closer than this threshold distance, they cease to develop as stomata. The arrested initials remain pluripotent, however, and differentiate as epidermal cells. Arrested initials acquire cellular characteristics of and expand as do epidermal cells, indicating that the temporal window for patterning is long enough for them to acquire a new cell fate.

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