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Investigation of the role of cell-cell interactions in division plane determination during maize leaf development through mosaic analysis of the *tangled* mutation

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SUMMARY

Most plant cells divide in planes that can be predicted from their shapes according to simple geometrical rules, but the division planes of some cells appear to be influenced by extracellular cues. In the maize leaf, some cells divide in orientations not predicted by their shapes, raising the possibility that cell-cell communication plays a role in division plane determination in this tissue. We investigated this possibility through mosaic analysis of the *tangled* (*tan*) mutation, which causes a high frequency of cells in all tissue layers to divide in abnormal orientations. Clonal sectors of *tan* mutant tissue marked by a closely linked albino mutation were examined to determine the phenotypes of cells near sector boundaries. We found that *tan* mutant cells always showed the mutant phenotype regardless of their proximity to wild-type cells, demonstrating that the wild-type *Tan* gene acts cell-autonomously in both lateral and transverse leaf dimensions to promote normally oriented divisions. However, if the normal division planes of wild-type cells depend on cell-cell communication involving the products of genes other than *Tan*, then aberrantly dividing *tan* mutant cells might send abnormal signals that alter the division planes of neighboring cells. The cell-autonomy of the *tan* mutation allowed us to investigate this possibility by examining wild-type cells near the boundaries of *tan* mutant sectors for evidence of aberrantly oriented divisions. We found that wild-type cells near *tan* mutant cells did not divide differently from other wild-type cells. These observations argue against the idea that the division planes of proliferatively dividing maize leaf epidermal cells are governed by short-range communication with their nearest neighbors.

Key words: Maize, Leaf development, Cell division, *tangled*

INTRODUCTION

During plant development, cell walls ensure that the relative positions of cells change little, if any. Consequently, the cellular organization of a plant tissue closely reflects the pattern of cell division during its development. In some species and tissue types, a virtually invariant sequence of oriented divisions elaborates a characteristic cell pattern. For example, stereotypical division patterns in the root tips of *Azolla* (a fern) and *Arabidopsis* (a dicot) establish the very regular arrangement of cells in these tissues (Gunning et al., 1978; Dolan et al., 1993; Dolan et al., 1994; Scheres et al., 1994; Kidner et al., 2000). In other tissues, such as the maize leaf, division pattern is more variable but nevertheless follows certain general rules that preserve a characteristic cellular organization (Langdale et al., 1989; Sylvester et al., 1990; Cerioli et al., 1994; Poethig and Szymkowiak, 1995). The scanning electron micrograph of a maize leaf primordium in Fig. 1A illustrates the pattern of proliferative epidermal cell divisions (divisions that produce most of the epidermal cells in the leaf and precede obvious signs of cellular differentiation). Leaf epidermal cells undergoing proliferative divisions are rectangular, with their long axes aligned with the long axis of the leaf. Within any given area of the primordium, some of the cells divide transversely and others divide longitudinally (placing the new wall perpendicular or parallel to the mother cell's long axis, respectively). The relative proportions of transverse and longitudinal divisions vary with developmental stage and position within the leaf (Sylvester et al., 1990; Poethig and Szymkowiak, 1995). The cell pattern of the mature maize leaf epidermis directly reflects this pattern of division: rectangular cells are organized into linear files, and cell walls are generally parallel or perpendicular to one another (Fig. 1C).

The lack of relative cell movement in plant tissues has led many to suppose that the generation of organ and plant shape during development also relies on precise control of cell division patterns. A different perspective has emerged from studies on *tangled* (*tan*) mutants of maize, in which the majority of cells divide in aberrant orientations throughout leaf development in all tissue layers, as illustrated for the epidermis in Fig. 1B (Smith et al., 1996). These aberrant divisions severely disrupt the cell pattern of mature *tan* mutant leaves, in which cells in all tissue layers are oddly shaped and chaotically arranged compared to those of wild-type leaves

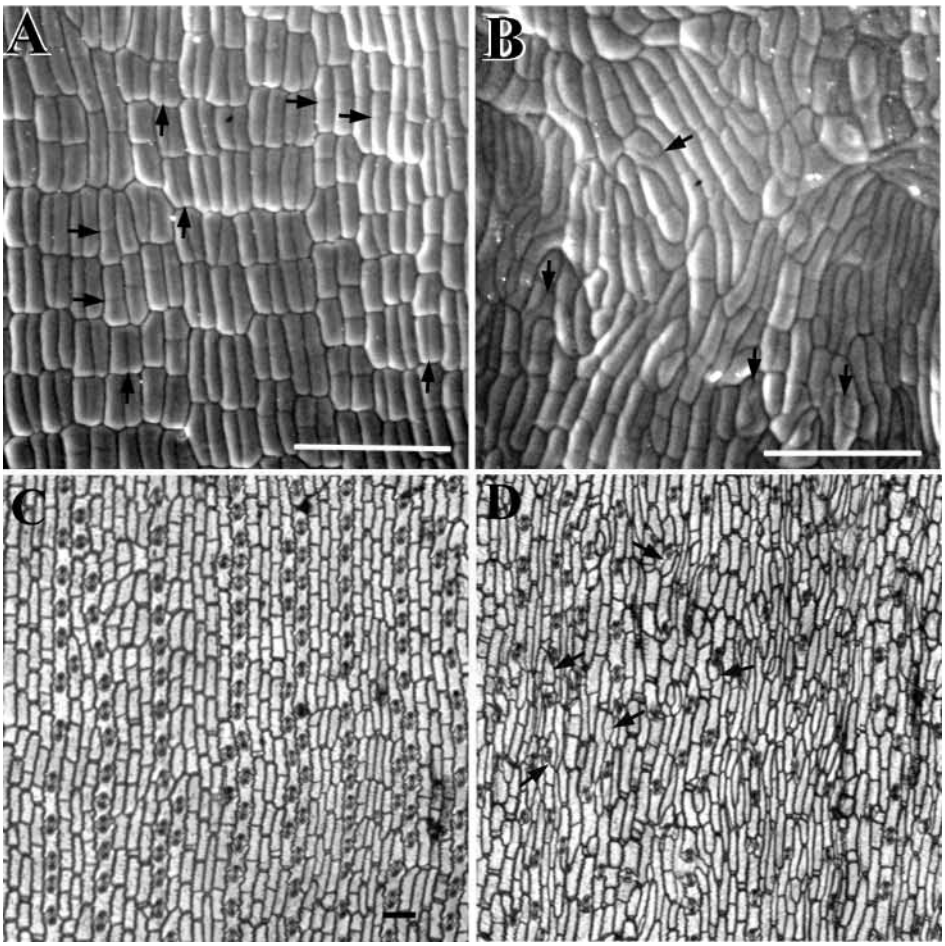

Fig. 1. Epidermal layers of wild-type and *tan* mutant leaves. (A) Scanning electron micrograph (SEM) of the surface of a wild-type maize leaf primordium. Shallow indentations in the leaf surface are indicative of recent cell divisions. Horizontal arrows point to several recent transverse divisions. Vertical arrows point to several recent longitudinal divisions. (B) SEM of the surface of a *tan* mutant leaf primordium. Arrows point to several recent abberant divisions. Scale bars in A and B: 100 μm. (C) Epidermal peel from mature, wild-type leaf illustrating a characteristic regular cell pattern. (D) Epidermal peel from a mature, *tan* mutant leaf illustrating a chaotic cell pattern. Arrows point to aberrantly positioned walls in interstomatal cells that were factored into calculation of abnormality index as described in Materials and Methods. Scale bar in C: 100 μm for C and D.

(shown for the epidermis, Fig. 1D). Nevertheless, the overall shape of *tan* mutant leaves is essentially normal, demonstrating that mechanisms governing leaf morphogenesis in maize can tolerate a high frequency of aberrantly oriented divisions (Smith et al., 1996).

The problem of how plant cells orient their divisions appropriately during development is one of longstanding interest, but remains largely unsolved (Smith, 2001). Over 100 years ago, plant biologists recognized relationships between cell shape and division plane that apply to most dividing cells. Hofmeister's rule states that new cell walls are usually formed in a plane perpendicular to the main axis of cell expansion – that is, perpendicular to the long axis of the mother cell (Hofmeister, 1863). Errera's rule states that the plane of division for most plant cells corresponds to the shortest path that will halve the volume of the parental cell (Errera, 1888). Although it is not fully known how a plant cell would be able to read its shape and divide accordingly, Lloyd and colleagues have proposed a model based on simple mechanical principles that could largely explain cells' ability to follow Hofmeister's and Errera's rules (Flanders et al., 1990; Lloyd, 1991).

However, not all cell division planes can be accurately predicted by these rules, and appear to be influenced by extracellular cues. For example, cells in the prospective leaf-forming region of the shoot apical meristem divide predominantly in different orientations than do those of similar shapes outside this region (Lyndon, 1972; Cunninghame and Lyndon, 1986). Observations on stomatal complex development in monocots suggest that newly formed guard mother cells signal their nearest neighbors to divide asymmetrically, forming small daughters adjacent to the guard mother cell that will become part of the complex (Stebbins and Shah, 1960; Stebbins and Jain, 1960). Another striking example emerges from laser ablation studies on developing *Arabidopsis* roots. When an individual cortex-endodermis initial cell within the root is ablated, a neighboring pericycle cell divides in an atypical orientation not predicted by its shape or position to produce a daughter that takes the place and assumes the fate of the ablated cell (van den Berg, 1995). The nature of the extracellular information these cells apparently respond to and how it is transmitted remain largely unknown, and may vary considerably in different situations. In animal cells, both cell shape and cell-cell communication can play important roles in determining planes of cell division (Goldstein, 2000). Though much remains to be learned about how cell-cell communication can direct the orientation of animal cell divisions, asymmetrically dividing cells in the early *C. elegans* embryo provide a relatively well understood example. Here, EMS cells signal neighboring P2 cells via the wingless/WNT pathway to re-orient their division planes (Schlesinger et al., 1999).

In the maize leaf primordium, as pointed out earlier, rectangular epidermal cells divide in both transverse and longitudinal orientations. For an elongated cell, a longitudinal

division plane is not predicted by Hofmeister's and Errera's rules. This raises the question of what role cell-cell interactions might play in division plane selection in this tissue. We have explored this question through a mosaic analysis of the *tan* mutation, in which we closely examined the boundaries of *tan* mutant sectors in otherwise wild-type leaves. If the *Tan* gene is involved in sending or controlling a signal that orients proliferative cell divisions, we would expect to find that it acts non cell-autonomously. That is, we would expect wild-type cells to 'rescue' genotypically mutant cells nearby so that they appear wild type. However, we found that the *tan* mutant phenotype is not rescued or influenced by adjacent wild-type cells, demonstrating that the wild-type *Tan* gene acts cell-autonomously to promote normal division orientations.

At sector boundaries, the juxtaposition of aberrantly divided mutant cells with wild-type cells gave us the opportunity to further explore the role of cell-cell communication in division plane determination by asking what impact mutant cells have on the divisions of neighboring wild-type cells. Although the *Tan* gene itself acts cell-autonomously, the proper orientation of proliferative divisions may nevertheless depend on cross-talk between adjacent, dividing cells involving the products of other genes. In this case, alterations in the signals sent by aberrantly dividing mutant cells could change the division planes of adjacent wild-type cells. Indeed, such cell-cell interactions have been invoked to explain how leaves of normal shape might form in *tan* mutants in spite of the high frequency of aberrantly oriented divisions. Meyerowitz (Meyerowitz, 1996) proposed that through local coordination of division orientations, *tan* mutant cells may divide so as to compensate for each others' mistakes, essentially correcting for each other to achieve an overall division pattern that permits the elaboration of normal leaf shape. In a fully mutant leaf, this idea is not readily testable because of the difficulty in recognizing such corrective divisions. However, corrective divisions in wild-type cells adjacent to mutant cells would be recognizable if they were oriented differently from other wild-type cell divisions. Our results show that proximity to *tan* mutant cells does not substantially alter the division orientations of wild-type cells. These observations argue against the idea that the division planes of proliferatively dividing maize leaf epidermal cells are governed by short-range communication with their nearest neighbors, and implicate spatial regulation of cell expansion rather than division as the primary determinant of leaf shape in both *tan* and wild-type leaves.

MATERIALS AND METHODS

Genetic stocks and generation of clonal sectors

Mutant plants homozygous for the *tan-Mu1* allele (Smith et al., 1996) were crossed to individuals heterozygous for the albino mutation, *w14*, obtained from the Maize Genetics Co-op Stock Center (Urbana, IL, USA). Doubly heterozygous progeny were outcrossed to wild type, and progeny from these crosses were selfed to identify individuals that had inherited *tan* and *w14* on the same chromosome as a result of recombination. One such recombinant chromosome was identified and propagated. To generate plants for mosaic analysis, individuals of the genotype *tan-w14*/*tan-W14*$^+$ were outcrossed to wild type. This way, we could be sure that *w14* could not be separated from *tan* through recombination, so *w14* was always inherited linked in *cis* with *tan*. 7,000 progeny seeds from these crosses (of which 50% were *tan-w14*/*Tan*$^+$-*W14*$^+$, and 50% were *tan-W14*$^+$/*Tan*$^+$-*W14*$^+$) were imbibed for 42-45 hours at 30°C and subsequently irradiated with 1000 R of gamma irradiation from a Cs source. Following irradiation, the seeds were hand planted in moist soil in the field at the University of California, San Diego during late spring or early summer. As the plants matured they were examined for white sectors, and 115 leaves with white sectors were recovered. Leaves containing white sectors were removed from the plants and screened for the presence of the *tan* phenotype in the epidermis by examining impressions of the leaf surface made in Loctite Superglue. This was necessary to identify useful sectors for three reasons. Because *W14*$^+$ is a few cM distal to *Tan*$^+$, some white sectors could be generated in which *tan* was not uncovered because of chromosome breakage between *W14*$^+$ and *Tan*$^+$. In addition, the *tan-Mu1* allele used for this study is dependent on *MuDR* activity for expression. Although the families chosen for irradiation were selected for the necessary *MuDR* activity, leaves in which this activity had been lost would not express the *tan* phenotype when *tan* is uncovered. Finally, spontaneous white sectoring sometimes occurs in our *Mutator* stocks, so some white sectors might not have been due to chromosome breakage uncovering *w14*. Since we found that the *tan* phenotype could be expressed in the epidermis even in sectors that were very small or lying over wild-type mesophyll, we could be sure that we were not excluding informative sectors by pre-screening for the presence of the *tan* phenotype. Twenty sectors that showed the *tan* phenotype in the epidermis were fully analyzed.

Analysis of sector composition

Hand-cut transverse sections of the sectors were made, mounted in water, and observed with a Nikon Eclipse E600 microscope equipped with fluorescence epi-illumination, using a standard rhodamine filter set. Images were acquired with a DAGE MTI CCD72 camera and digitized with a Scion LG3 framegrabber using Scion Image 1.62. Images were collected under both bright-field and epifluorescence conditions to record the distribution of chlorophyll-containing cells. In instances where multiple images needed to be taken to span the entire sector, a composite of adjacent cross sections was created using Adobe Photoshop 4.0.1.

Epidermal peels were also prepared from a portion of each sector (immediately adjacent to the location of the hand cross sections) as described previously (Gallagher and Smith, 1999). Prior to fixation each sector boundary was marked with a Sharpie ink pen so that it could later be aligned with the corresponding hand section; because only guard cells in the epidermis contain chloroplasts, boundaries marked with ink represented lateral boundaries in the mesophyll. For each sector, bright-field and epifluorescence images of epidermal peels were collected as described above and assembled into a composite image of the entire sector in surface view. Guard cells showing chlorophyll autofluorescence were marked on the composite image. Information from cross sections and epidermal peels was compiled to make a complete illustration of each sector showing both mesophyll and epidermal composition as shown in Fig. 2.

Quantitative analysis of wall orientations

To determine whether wild-type cells affect division planes of mutant cells or vice versa, a quantitative analysis of wall orientations was performed on all sectors for which the boundary region in the epidermis was sufficiently clear and did not coincide with an underlying major vein. Cells flanking sector boundaries were scored individually according to whether they had one or more oblique or aberrantly localized walls indicative of abnormal planes of cell division. In stomatal files, each stomate was scored as normal if the interstomatal cells above and below it had no aberrant walls. If a stomate had at least one adjacent interstomatal cell with at least one aberrant wall, or was adjacent to more than two interstomatal cells, it was scored as abnormal. Examples of aberrantly positioned walls that

would have resulted in the associated stomate being scored as abnormal are indicated by arrows in Fig. 1D. The abnormality index of a stomatal file was calculated as the proportion of stomata in the file that were abnormal according to these criteria. The abnormality index for non-stomatal files was calculated simply as the proportion of all cells in the file having one or more oblique or aberrantly localized walls. In addition to analyzing cell files near sector boundaries, cell files far from boundaries were also analyzed for purposes of comparison. These were always at least 10 files away from the nearest epidermal sector boundary.

Scanning electron microscopy

Scanning electron micrographs of the surface of wild-type and *tan* mutant maize leaf primordia were prepared as described previously (Smith et al., 1996).

RESULTS

Construction and analysis of sectors

For mosaic analysis, plants heterozygous for *tan* and a closely linked, cell-autonomous albino mutation, *w14*, were gamma irradiated to induce chromosome breaks. Occasional loss of the portion of chromsome 6 carrying wild-type alleles of *Tan* and *W14* resulted in the formation of albino-marked sectors of *tan* mutant tissue (for details see Materials and Methods). We identified and fully analyzed 20 such sectors. Complete analysis of the sectors involved making hand-cut cross sections and epidermal peels. Cross sections allowed us to determine sector composition and lateral boundaries in the internal tissue layers of the leaf. Epidermal peels allowed us to locate lateral sector boundaries in the epidermis and clearly see the *tan* phenotype. Information from both cross sections and epidermal peels was used to assemble a complete characterization of each sector's composition, as shown in Fig. 2 for a hypothetical sector.

In the epidermis, only guard cells could be scored as *w14* or *W14*$^+$, because these are the only epidermal cells containing mature chloroplasts. Consequently, lateral sector boundaries in the epidermis could be located to the interval between one row of stomata containing chloroplasts and another without chloroplasts, which could be distinguished by the presence or absence of chlorophyll autofluorescence. Examples of lateral boundaries in the epidermis are seen in Fig. 3A-D where the white asterisks indicate wild-type stomata containing chloroplasts and the black asterisks indicate mutant stomata lacking chloroplasts. In each case, a lateral boundary is located to the interval between the wild-type and mutant stomatal files. As illustrated in Fig. 3A-D, the number of cell files separating wild-type and mutant stomata fluctuates along the length of the sector boundary. Quantitative analysis (Fig. 4) showed that the number of files separating wild-type and mutant stomata was most often 2, 3 or 4 (73% of the time). Thus, most of the time, we could be sure that the true distance from the sector boundary to the nearest marked stomatal file was no more than 4 cells. 29% of the time, the distance was no more than 2 cells. Examples of wild-type and mutant stomata separated by 2 or fewer cells are indicated by numbered black arrowheads in Fig. 3A-D.

The genotypes of internal tissue layers can be viewed in leaf cross sections as illustrated in Fig. 2. A lateral boundary in the epidermis may coincide with a lateral boundary in the mesophyll (Fig. 2, arrow 2). More often, however, epidermal lateral boundaries overlie either wild-type or mutant mesophyll (Fig. 2, arrows 1 and 3). In combination with data from analysis of epidermal peels, cross sections also reveal the presence of transverse sector boundaries, defined here as boundaries between epidermal and mesophyll layers of different genotypes. These can consist of wild-type epidermis overlying mutant mesophyll (Fig. 2B) or mutant epidermis overlying wild-type mesophyll (Fig. 2D). From the 20 sectors chosen for analysis, we examined 56 lateral boundaries, 13 transverse boundaries with wild-type epidermis over mutant mesophyll, and 33 transverse boundaries with mutant epidermis over wild-type mesophyll.

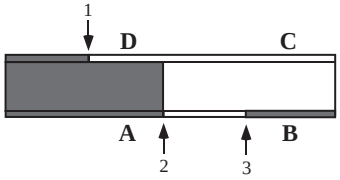

Fig. 2. Diagram of a hypothetical leaf in cross-sectional view illustrating all possible types of sector boundaries. Upper and lower epidermises are demarcated by closely spaced horizontal lines, and are separated by multiple mesophyll layers. Shaded areas represent wild-type tissue; unshaded areas represent *w14*-marked *tan* mutant tissue. Wild-type epidermal cells may overlie wild-type mesophyll (A) or mutant mesophyll (B). Mutant epidermal cells may overlie mutant mesophyll (C) or wild-type mesophyll (D). Transverse sector boundaries between the epidermis and mesophyll occur when wild-type epidermal cells overlie mutant mesophyll (e.g., area B) or when mutant epidermal cells overlie wild-type mesophyll (e.g., area D). Lateral sector boundaries in the epidermis are represented by numbered arrows. A lateral boundary in the epidermis may coincide directly with a lateral boundary in the mesophyll (arrow 2). It may also occur over either wild-type (arrow 1) or mutant (arrow 3) mesophyll.

Tan acts cell-autonomously

Inspection of mutant epidermal cells near wild-type epidermal cells or overlying wild-type mesophyll allowed us to determine whether or not *tan* is cell-autonomous. When lateral sector boundaries in the epidermis and mesophyll coincide, a sharp transition from wild-type to *tan*-appearing cells is seen between the wild-type and mutant stomatal rows (Fig. 3A,B). Lateral sector boundaries in the epidermis also show the same sharp transition when they overlie wild-type mesophyll (Fig. 3C) or mutant mesophyll (Fig. 3D). Thus, we observed that *tan* mutant cells always showed the mutant phenotype, even when close to wild-type cells. In fact, many examples of aberrantly divided (presumably mutant) cells were observed in the boundary region immediately adjacent to marked, wild-type stomatal files (black arrows in Fig. 3A-C). Moreover, we found that mutant epidermis overlying wild-type mesophyll (Fig. 3C) appears to have as severe a *tan* phenotype as mutant epidermis overlying mutant mesophyll (Fig. 3A,B). The fact that mutant epidermal cells are not phenotypically rescued by underlying or adjacent wild-type cells indicates that *tan* is cell-autonomous in both lateral and transverse dimensions.

Although visual inspection of sectors revealed no effect of nearby wild-type cells on the phenotypes of mutant cells, we considered the possibility that there could be a small effect not apparent from casual observation. To do this, we carried out a quantitative analysis comparing the frequency of aberrantly oriented walls in mutant cells near wild-type cells with that in

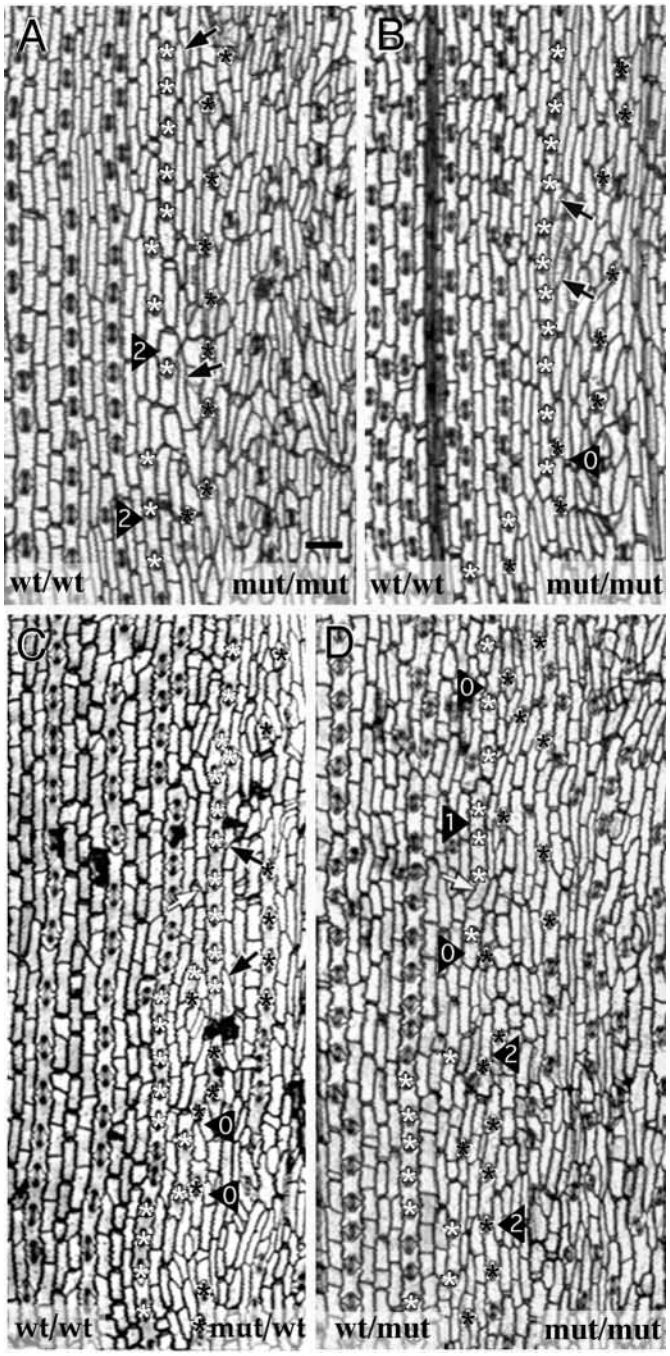

Fig. 3. Phenotype observed at lateral sector boundaries in the epidermis. Epidermal and mesophyll genotypes are indicated to the left and right of each sector boundary (i.e., wt/wt means the epidermis and underlying mesophyll are both wild type; wt/mut means the epidermis is wild type and the underlying mesophyll is mutant, etc.). White asterisks indicate wild-type guard cells containing chloroplasts. Black asterisks indicate mutant guard cells lacking chloroplasts. Black arrows indicate oblique cell walls immediately adjacent to marked, wild-type cells. Note that the wild-type cells adjacent to these walls are of normal shape. White arrows indicate aberrantly divided wild-type cells. For regions where the interval between wild-type and mutant stomatal files is 2 or fewer cell files, numbered black arrowheads indicate the distance between the two files. (A,B) Lateral sector boundaries in the epidermis coinciding with lateral boundaries in the underlying mesophyll. Note the sharp transition between wild-type and mutant-appearing cells regardless of the distance between the stomatal files marking the boundary region. (C) Lateral sector boundary in the epidermis overlying wild-type mesophyll. Note that the phenotypes of mutant cells overlying wild-type mesophyll (lower right quadrant) are comparable to those of mutant cells overlying mutant mesophyll in A, B and D. (D) Lateral sector boundary in the epidermis overlying mutant mesophyll. Note that wild-type epidermal cells overlying mutant mesophyll (left half) appear as regular as those overlying wild-type mesophyll in A, B and C. Scale bar in A:100 μm for A-D.

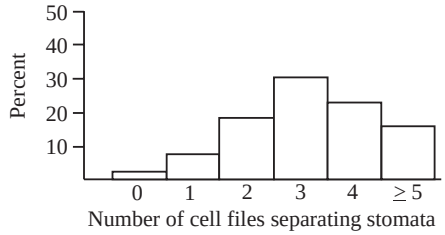

Fig. 4. Proximity of mutant and non-mutant stomatal files marking the boundary region. The number of cell files separating individual wild-type stomata from the nearest mutant stoma was counted for a total of 453 stomatal pairs. For 0 files, *n*=13; 1 file, *n*=36; 2 files, *n*=83; 3 files, *n*=141; 4 files, *n*=107; 5 or more files, *n*=73.

mutant cells far from wild-type cells. An 'abnormality index' was calculated for selected cell files, which reflected the proportion of cells with oblique or aberrantly located walls, indicative of abnormal planes of cell division (see Materials and Methods for details). As shown in Fig. 5A, abnormality indexes for mutant stomatal files near boundaries with wild-type epidermis or overlying wild-type mesophyll were not significantly different from that for mutant stomatal files nowhere near wild-type cells.

Because stomatal rows tend to be the most ordered cell files in mutant epidermis, we also analyzed non-stomatal mutant files near sector boundaries. To be certain of choosing genotypically mutant files as close as possible to sector boundaries, we analyzed those immediately adjacent to marked, mutant stomatal files on the side opposite the sector boundary. For example, for the sector boundaries illustrated in Fig. 3, this would be the cell file immediately to the right of the stomatal file marked with black asterisks. As shown in Fig. 5B, the abnormality indexes for mutant, non-stomatal files near wild-type epidermal cells or overlying wild-type mesophyll were not significantly different from the abnormality index for mutant, non-stomatal files far from wild-type cells. Thus, results of visual and quantitative analyses concur in showing that *tan* is cell-autonomous in both the lateral and transverse dimensions.

Mutant cells do not cause nearby wild-type cells to divide aberrantly

Since we found that *Tan* acts cell-autonomously, we could also ask how the proximity of mutant cells might affect the divisions of wild-type cells. Do wild-type cells divide in aberrant orientations to somehow compensate for or respond to abnormally dividing mutant cells nearby? As discussed in more detail in the Introduction, this might occur if division planes in

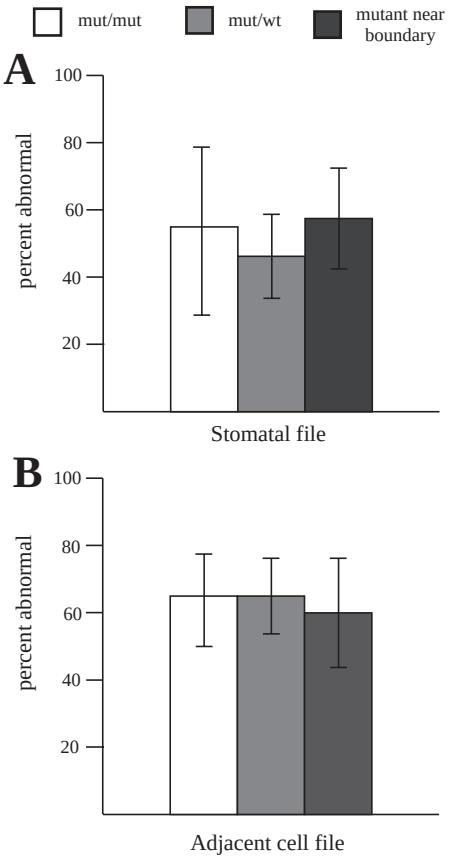

Fig. 5. Abnormality index calculated for mutant epidermal cells overlying mutant mesophyll or wild-type mesophyll, or near a lateral sector boundary in the epidermis (see Materials and Methods for methodology). (A) Stomatal files. (B) Adjacent, non-stomatal files. Error bars show standard deviations.

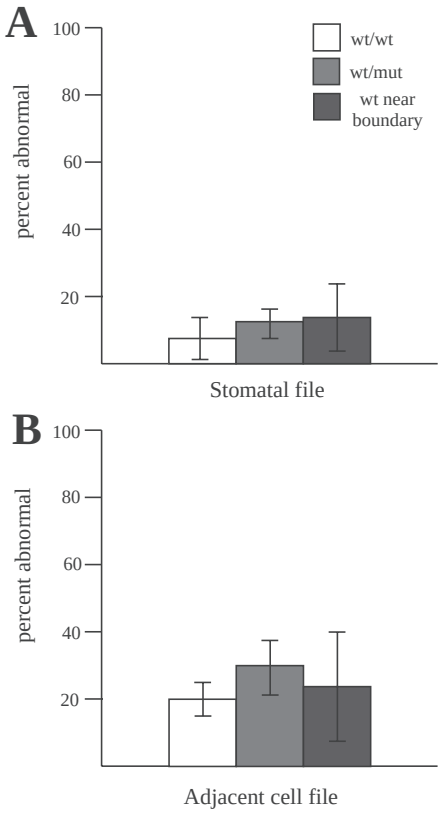

Fig. 6. Abnormality index calculated for wild-type epidermal cells overlying wild-type mesophyll or mutant mesophyll, or near a lateral sector boundary in the epidermis (see Materials and Methods for methodology). Note that for 5 of the 6 transverse boundaries included in this analysis having wild-type epidermal cells overlying mutant mesophyll, all mesophyll layers were mutant. (A) Stomatal files. (B) Adjacent, non-stomatal files. Error bars show standard deviations.

normal leaf tissue are determined through some form of cell-cell communication involving the products of genes other than *Tan*. We found that occasionally, aberrant cell divisions occurred in wild-type epidermal cells adjacent to or overlying mutant cells (e.g., white arrows Fig. 3C,D). These aberrant divisions were rare, however, and did not usually occur near other improper divisions. Even in areas where wild-type and mutant stomata were no more than 2 files apart, the wild-type cells appeared to have divided normally (Fig. 3A-D). In fact, normally divided wild-type cells were often observed directly adjacent to aberrantly divided (presumably mutant) cells in the boundary region (e.g., black arrows Fig. 3A-C). Thus, visual inspection indicated that wild-type cells do not divide aberrantly under the influence of nearby mutant cells.

To determine whether there could be a small effect of mutant cells on wild-type cells, we performed a quantitative analysis of abnormal wall orientations in wild-type cells near mutant cells as described earlier. As shown in Fig. 6A, the abnormality indexes for wild-type stomatal files near boundaries with mutant epidermal cells or overlying mutant mesophyll were not significantly different from the index for wild-type stomatal files far from mutant cells. Moreover, the abnormality indexes for wild type, non-stomatal files near boundaries with mutant epidermal cells (such as those immediately to the left of the files marked with white asterisks in Fig. 3) or overlying mutant mesophyll were also not significantly different from the index for wild type, non-stomatal files far from mutant cells (Fig. 6B). Thus, both visual and quantitative analyses showed that the division planes of wild-type cells are not substantially altered by the proximity of mutant cells.

DISCUSSION

In this study, we used mosaic analysis to investigate the contributions of cell-cell communication to determination of cell division planes in developing maize leaves. The first question we asked was whether the maize *Tangled* (*Tan*) gene acts cell-autonomously or non cell-autonomously. We found that *tan* cells display the mutant phenotype even when they are in close proximity to wild-type cells. This is true for mutant epidermal cells overlying wild-type mesophyll cells, as well as for mutant epidermal cells near wild-type epidermal cells. Thus, we conclude that the wild-type *Tan* gene acts cell-autonomously in both lateral and transverse leaf dimensions. The fact that even the smallest sectors of mutant tissue examined (a few millimeters wide and confined to a single leaf) show a fully mutant phenotype indicates that the effects of *tan* on leaf cell division (Smith et al., 1996; Cleary and Smith,

1998) are not an indirect consequence of abnormal events occurring much earlier in development, at or before initiation of the leaf primordium. Rather, these results argue that *Tan* acts locally to promote normally oriented divisions in the leaf on a cell-by-cell basis. These conclusions are consistent with what we know about *Tan* function at the molecular level. The *Tan* gene is expressed in mitotic but not post-mitotic leaf cells; it encodes a highly basic ~43 kDa protein that can bind to microtubules in vitro and belongs to a family of proteins that are preferentially associated with the cytoskeleton in dividing cells (Smith et al., 2001). Although the mechanism by which TAN protein helps to orient cytoskeletal arrays during cell division remains to be elucidated, our results point to an intracellular function for this protein and do not suggest that it functions in cell-cell communication.

The cell-autonomy of *Tan* gene function allowed us to address the additional question of how *tan* mutant cells affect the divisions of neighboring wild-type cells. If division planes in the developing leaf epidermis are governed by short-range cross-talk between neighboring cells involving genes other than *Tan*, then wild-type cells might respond to the abnormal divisions of adjacent *tan* mutant cells by dividing differently themselves. Such local interactions allowing cells to compensate for each other's aberrant divisions have been proposed to explain how normal leaf shape can be acquired in *tan* mutant leaves (Meyerowitz, 1996). Therefore, we closely examined wild-type epidermal cells neighboring mutant cells for evidence that aberrant divisions had taken place. We found that there was no significant increase in the frequency of abnormally positioned walls in wild-type epidermal cells overlying mutant mesopyll cells or adjacent to mutant epidermal cells. Owing to the adjustments in wall orientation that could take place during postmitotic cell expansion, we cannot rule out the possibility that minor aberrations in wall orientation were present in wild-type cells immediately following division but were undetectable at maturity. However, our results indicate that the division planes of wild-type cells in close proximity to aberrantly dividing mutant cells were not substantially altered.

This observation lends support to the previously proposed view that the generation of normal leaf shape in both *tan* and wild-type leaves is achieved primarily through spatial control of cell expansion rather than cell division (Smith et al., 1996; Cleary and Smith, 1998; Reynolds et al., 1998). Thus, if mechanisms responsible for orienting cell expansion can operate on abnormally shaped cells to orient their expansion appropriately relative to the leaf as a whole, then a high frequency of abnormally oriented divisions need not alter the overall pattern of leaf growth so long as there is a sufficient number of cells to support growth in the appropriate directions. Consistent with this proposal, a recent study has shown that localized induction of expansin gene expression within tobacco leaf primordia can induce dramatic changes in the pattern of leaf morphogenesis (Pien et al., 2001). This suggests that the regional variations in wall extensibility within the leaf primordium governed by the pattern of expansin gene expression could play a primary role in determining leaf shape.

Our observation that the division planes of wild-type epidermal cells are unperturbed by aberrantly oriented divisions of adjacent, *tan* mutant cells argues against the idea that the division planes of proliferatively dividing maize leaf epidermal cells are governed by short-range communication with their nearest neighbors. However, this does not mean that cell-cell interactions play no role in division plane determination in this tissue. While the majority of epidermal cells in the maize leaf primordium may choose transverse division planes simply because of their elongated shapes, some choose longitudinal division planes that are not predicted by shape according to Hofmeister's and Errera's rules. Thus, the high frequency of longitudinally oriented divisions in this tissue remains to be explained, and may involve extracellular influences of some kind. One possibility is that cues stimulating cell expansion in the width dimension can also override the 'default' choice of a transverse division plane to produce longitudinal divisions. Another intriguing possibility is suggested by experiments demonstrating that application of a compressive force to callus cultures as well as to single cells in suspended in semi-solid medium can alter cell division planes (Lintilhac and Vesecky, 1984; Lynch and Lintilhac, 1997). Thus, cell-cell interactions of a mechanical nature within the developing leaf primordium may cause some cells to divide longitudinally. According to either of these explanations, defects in cell plate-orienting mechanisms in *tan* mutant cells could account for their high frequency of aberrantly oriented divisions without predicting that these aberrant divisions would interfere with the divisions of adjacent, wild-type cells.

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REFERENCES

Cerioli, S., Marocco, A., Maddaloni, M., Motto, M. and Salamini, F. (1994). Early events in maize leaf epidermis formation as revealed by cell lineage studies. *Development* **120**, 2113-2120.

Cleary, A. L. and Smith, L. G. (1998). The *Tangled1* gene is required for spatial control of cytoskeletal arrays associated with cell division during maize leaf development. *Plant Cell* **10**, 1875-1888.

Cunninghame, M. E. and Lyndon, R. F. (1986). The relationship between the distribution of periclinal cell divisions in the shoot apex and leaf initiation. *Ann. Bot*. **57**, 737-746.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. and Scheres, B. (1993). Cellular organization of the *Arabidopsis thaliana* root. *Development* **119**, 71-84.

Dolan, L., Duckett, C. M., Grierson, C., Linstad, P., Schneider, K., Lawson, E., Dean, C., Poethig, S. and Roberts, K. (1994). Clonal relationships and cell patterning in the root epidermis of *Arabidopsis*. *Development* **120**, 2465-2474.

Errera, L. (1888). Über Zellformen und Siefenblasen. *Botanisches Centralblatt* **34**, 395-399.

Flanders, D. J., Rawlins, D. J., Shaw, P. J. and Lloyd, C. W. (1990). Nucleus-associated microtubules help determine the division plane of plant epidermal cells: avoidance of four-way junctions and the role of cell geometry. *J. Cell Biol.* **110**, 1111-1122.

Gallagher, K. and Smith, L. G. (1999). *discordia* mutations specifically misorient asymmetric cell divisions during development of the maize leaf epidermis. *Development* **126**, 4623-4633.

Goldstein, B. (2000). When cells tell their neighbors which direction to divide. *Dev. Dynam.* **218**, 23-29.

Gunning, B. E. S., Hughes, J. E. and Hardham, A. R. (1978). Formative and proliferative cell divisions, cell differentiation and developmental changes in the meristem of *Azolla* roots. *Planta* **143**, 121-144.

Hofmeister, W. (1863). Zusatze und Berichtigungen zu den 1851 veröffentlichen Untersuchungengen der Entwicklung höherer Kryptogamen. *Jahrbucher für Wissenschaft und Botanik* **3**, 259-293.

Kidner, C., Sundaresan, V., Roberts, K. and Dolan, L. (2000). Clonal analysis of the *Arabidopsis* root confirms that position, not lineage, determines cell fate. *Planta* **211**, 191-199.

Langdale, J. A., Lane, B., Freeling, M. and Nelson, T. (1989). Cell lineage analysis of maize bundle sheath and mesophyll cells. *Dev. Biol.* **133**, 128-139.

Lintilhac, P. M. and Vesecky, T. B. (1984). Stress-induced alignment of division planes in plant tissues grown in vitro. *Nature* **307**, 363-364.

Lloyd, C. W. (1991). How does the cytoskeleton read the laws of geometry in aligning the division plane of plant cells? *Development* **Suppl. 1**, 55-65.

Lynch, T. M. and Lintilhac, P. M. (1997). Mechanical signals in plant development: a new method for single cell studies. *Dev. Biol.* **181**, 246-256.

Lyndon, R. F. (1972). Leaf formation and growth of the shoot apical meristem. *Physiologie Vegetale* **10**, 209-222.

Meyerowitz, E. M. (1996). Plant development: local control, global patterning. *Curr. Opin. Genet. Dev.* **6**, 475-479.

Pien, S., Wyrzykowska, J., McQueen-Mason, S., Smart, C. and Fleming, A. (2001). Local expression of expansin induces the entire process of leaf development and modifies leaf shape. *Proc. Natl. Acad. Sci. USA* **98**, 11812-11817.

Poethig, R. S. and Szymkowiak, E. J. (1995). Clonal analysis of leaf development in maize. *Maydica* **40**, 67-76.

Reynolds, J. O., Eisses, J. F. and Sylvester, A. W. (1998). Balancing cell division and expansion during maize leaf morphogenesis; analysis of the mutant, *warty1*. *Development* **125**, 259-268.

Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C. and Weisbeek, P. (1994). Embryonic origin of the *Arabidopsis* primary root and root meristem initials. *Development* **120**, 2475-2487.

Schlesinger, A., Shelton, C. A., Maloof, J. N., Meneghini, M. and Bowerman, B. (1999). Wnt pathway components orient a mitotic spindle in the early *Caenorhabditis elegans* embryo without requiring gene transcription in the responding cell. *Genes Dev.* **13**, 2028-2038.

Smith, L. G. (2001). Plant cell division: building walls in the right places. *Nature Rev. Molec. Cell Biol.* **2**, 33-39.

Smith, L. G., Hake, S. and Sylvester, A. W. (1996). The *tangled1* mutation alters cell division orientations throughout maize leaf development without altering leaf shape. *Development* **122**, 481-489.

Smith, L. G., Gerttula, S. M., Han, S. and Levy, J. (2001). TANGLED1: a microtubule binding protein required for spatial control of cytokinesis in maize. *J. Cell Biol.* **152**, 231-236.

Stebbins, G. L. and Jain, S. K. (1960). Developmental studies of cell differentiation in the epidermis of monocotyledons. I. *Allium*, *Rheo* and *Commolina*. *Dev. Biol.* **2**, 409-426.

Stebbins, G. L. and Shah, S. S. (1960). Developmental studies of cell differentiation in the epidermis of monocotyledons I. Cytological features of stomatal development in the *Gramineae*. *Dev. Biol.* **2**, 177-200.

Sylvester, A. W., Cande, W. Z. and Freeling, M. (1990). Division and differentiation during normal and *liguleless1* maize leaf development. *Development* **110**, 985-1000.

van den Berg, C., Willemsen, V., Hage, W., Weisbeek, P. and Scheres, B. (1995). Cell fate in the *Arabidopsis* root meristem determined by directional signaling. *Nature* **378**, 62-65.