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Balancing division and expansion during maize leaf morphogenesis: analysis of the mutant, *warty-1*

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SUMMARY

Cell division and expansion are growth events that contribute to the developing shape, or morphogenesis, of a plant. Division and expansion are coordinated to the extent that plant organs, such as leaves, generally portray a predictable cellular pattern. To dissect the relationship between division and expansion, and to test for the role of each during morphogenesis, we have identified a recessive mutation *warty-1* that produces a primary defect in cell size and shape in mutant leaves. *Warty-1* mutant plants are similar to non-mutant siblings in terms of flowering time, overall plant size and leaf shape. Mature adult leaves have raised warts, consisting of excessively enlarged cells, that appear in patchy distribution throughout the blade. Cell wall deposition is abnormal or incomplete, suggesting cytokinesis is also affected, either directly or indirectly. Cells first increase in size at specific positions, which correspond to predictable cell dimensions of a developing 1 cm leaf. Once mutant cells exceed 133% normal size, cytokinesis becomes abnormal. As differentiation progresses, cells that appear normal in the mutant are actually dividing faster and are smaller than comparable cells in non-mutant siblings. These results suggest that (1) cells may compensate for growth defects by altering their cell cycle and that (2) proper execution of cytokinesis may require that cell size ratios are properly maintained.

Keywords: Leaf development, Maize, Cell division, Cell expansion, *warty-1*

INTRODUCTION

Plants elaborate leaves in a repeatable, consistent fashion yet different leaf forms are thought to be generated by processes that are mechanistically similar at the cellular level. Cell patterns in a mature organ represent a history of cell behavior during development: cells divide and expand differentially at particular orientations and rates. An ongoing question concerns the precise role of these predictable cellular patterns in organ morphogenesis; in other words, to what extent are leaf morphogenesis and the patterning of cells interdependent? Furthermore, the issue of how cells communicate to coordinate local growth remains incompletely understood. To consider these questions of cell communication and of interdependency between cell pattern and morphogenesis, it is important to understand the interaction between division and expansion.

Recent studies show that morphogenesis in plants is not dependent on precise division orientation or rate, thus bringing into question the view that form in plants is determined by precisely executed cell divisions (Smith et al., 1996; Smith, 1996; Sylvester et al., 1996). For example, particular orientations of cell divisions are reduced in number without influencing the shape or development of mutant *tangled-1* (*tan-1*) leaves (Smith et al., 1996). Defective pre-prophase bands will alter cell pattern without affecting differentiation, albeit organ shapes are altered (Traas et al., 1995). Transgenic tobacco plants that express low levels of the *cdc2* protein divide slower but grow normally (Hemerly et al., 1995). These studies support the view that morphogenesis can be uncoupled from an organized cell pattern, as generated by altered cell divisions. The discovery that cell pattern can be dissociated from morphogenesis raises intriguing questions about the mechanism of generating shape changes in a plant (Kaplan and Hageman, 1991; Cooke and Lu, 1992; Green 1994; Smith et at., 1996; Meyerowitz, 1996).

The role of cell expansion per se in morphogenesis has not been studied extensively. Both division and expansion affect the final cell pattern in an organ such that the direction and extent of one event likely influences the other. Cell division and expansion are indeed so intimately related that it is often difficult to disengage the two growth events for experimental study. If division orientation is influenced by the preceding shape of a cell, then disruption of one may be compensated for by changes in the other. For example, the absence of oriented divisions in some mutants could be balanced by reoriented direction or changed extent of cell expansion, thus maintaining normal overall growth patterns of the whole organ. Cells could thus compensate for local disturbances, and therefore retain the proper balance of cells required for morphogenesis. Such cross-talk between cells has been considered by others (Green,

1994; Meyerowitz, 1996) and tested by Freeling (1992), who showed that leaves could adjust shape when adjoining clonal sectors grow at different rates.

The balance between division and expansion is likely dictated by as yet unidentified regulators. To identify such regulators, it will be useful to find mutations that cause phenotypes in which division and expansion are distinguishable. Maize is essential for this type of study because morphogenesis is well-understood and cell arrangement is highly axial (Sharman, 1942; Poethig, 1984; Sylvester et al., 1990; Poethig and Szymkowiak, 1995). Most new cross walls are deposited either parallel (longitudinal) to the leaf axis or perpendicular (transverse) to the leaf axis. Similarly, cells expand unidirectionally resulting in the typical orderly and axial distribution of cells in the grass leaf (Poethig, 1984; Sylvester et al., 1990; Poethig and Szymkowiak, 1995; reviewed in Sylvester et al., 1996). For mutant screening, cells of unusual sizes and shapes can be identified readily, due to this regularity of cell pattern. The mutant phenotype can be characterized and the gene tagged with transposons for cloning and molecular analysis. Taking this approach, we describe here a mutation *warty-1* (*wty-1*) that was identified in a cellular screening of EMS (ethyl methane sulfonate) mutagenized M_2 plants. *Wty-1* mutants have abnormally expanded cells and thus are useful for teasing apart genetically the relationships among cell division, expansion and organ morphogenesis.

This paper describes the basic phenotype of *wty-1* mutant plants and considers whether cells compensate to maintain normal morphogenesis in the face of abnormal cell pattern. The cell expansion defect in mutant *wty-1* leaves appears in patches (warts) throughout the leaf blade. We show that *wty-1* mutants are normal in leaf proportions, organ shape, flowering time. Cells in warts are disorganized and the primary mutant defect is initially in the extent, not necessarily orientation, of cell expansion. Select cells exceed a normal size range and the overly enlarged cells subsequently divide abnormally and continue to expand excessively. Cells that appear normal in size and shape in the mutant divide more rapidly and expand less than either adjacent abnormally sized mutant cells or comparably positioned cells in non-mutant siblings.

MATERIALS AND METHODS

The *wty-1* allele was identified in a field screen of M_2 seed. Pollen was originally mutagenized with EMS, the progeny were selfed and the resulting M_2 seed were kindly provided by Dr Gerry Neuffer. Developmental analyses were conducted on selfed heterozygotes that had been introgressed three generations into inbred line ND-101. Complementation tests were conducted between *wty-1* and two mutations, *bumpy*-1 (*bum-1*) and *rough-lineate-1* (*rl-1*), each derived from independent EMS mutagenesis treatments. *bum-1* seeds were generously provided by Barbara Lane and Dr Neelimha Sinha. *rl-1* seeds were generously provided by Dr Gerry Neuffer.

The first five to six leaves of maize are produced during the juvenile stage of the plant's development, and differ in morphology and senescence rate from the adult leaves produced subsequenty (for a fuller description see Sylvester et al., 1990). Mature, non-growing adult leaves were characterized using light microscopy. Whole leaves were cleared in 3:1 ethanol:acetic acid, rehydrated and stained in 0.01% toluidene-blue-O (TBO) at pH 4.5. For sectioning, leaves were vacuum infiltrated overnight with FAA (10% formalin, 5% acetic acid, 50% alcohol), dehydrated in a graded alcohol series, embedded in Paraplast, sectioned at 8 μm thickness and stained with 1% TBO at pH 4.5. Alternatively, leaves were fixed in Flemming's Fluid (Clark, 1981) for 24 hours, dehydrated in a graded alcohol series, embedded in JB-4 medium (Polysciences, Inc), sectioned at 2 μm thickness and stained with 1% TBO.

Changes in leaf proportions were compared in mutant and non-mutant siblings. Segregating seed were grown in standard greenhouse conditions of 24°C with 16L:8D photoperiod. Seedlings were dissected at approximately the 6 leaf stage, identified by the 6th leaf just emerging from the leaf furl. Blade and sheath lengths were measured for each leaf as it was removed and was recorded for three mutant and three non-mutant individuals from the same family. Blade to sheath ratios in sequential leaves were thus used as a measure of the changing proportions of developing leaf regions.

Immature growing adult leaves were obtained from 8 leaf stage plants, identified by the presence of the 8th leaf just emerging from the leaf furl. Plants were dissected to reveal a 1 cm long leaf 9 or 10 (immature adult leaves). Three mutant and three non-mutant siblings were used. The leaf was removed from the shoot apex, cut in half at the midrib and unrolled to reveal the adaxial surface. A dental impression medium (Reprosil medium body, Patterson Dental Supply Co) was applied to the entire surface and removed after polymerization in 3-5 minutes. A thin coat of nail polish (Wonder Wear, base coat, Revlon) was applied to the impression, allowed to dry and then gently peeled off and placed face-up on a glass slide. A cover-slip was positioned, without mounting medium, over the nail polish cast and stabilized with a drop of nail polish. Casts of entire leaves were examined using a standard light microscope (Zeiss Axiophot), using false dark-field optics to reveal cell walls in high contrast or DIC optics to examine cells in relief. Images were recorded using a CCD video-camera (Hamamatsu model XC-77) and analyzed with NIH Image 1.61 using a Macintosh Centris 650 computer equipped with a frame-grabber (Scion Lg3).

Cell axiality is maintained in the *wty-1* mutant, allowing a comparison of cell orientation relative to the local leaf axis. Local leaf axes were determined by tracing back the trajectory of veins or of developing prickle hairs in the differentiation zone, which parallel the underlying veins. An image field was oriented to the local leaf axis. Cell pairs that had recently divided were first identified as described previously (Sylvester et al., 1990). Recently deposited cell plates are shallower and thinner than the adjoining parent cell wall (see Figs 5, 7).

Recent cell pairs were classified as 'longitudinal' if derived from longitudinal divisions (i.e the new cell plate is parallel to the local long axis of the leaf) or as 'transverse' if derived from a transverse division (i.e the new cell plate is perpendicular to the local long axis of the leaf). Incomplete or abnormal cell divisions were classified as 'abnormal longitudinal' because in all cases the abnormality was closest to longitudinal in orientation. Cell length and width were then measured in the classified cell pairs in each sub-zone separately (see Fig. 7). Generally, 20-30 cell pairs were visible per captured image. Several fields of cells could be analyzed per sub-zone per leaf. These were summed per individual and means of three individuals are displayed in the graphs. Cell pair length was plotted against width to give size ratios of the pairs classified as transverse or longitudinal.

Cells sizes and division rates were also quantified in the differentiation zone by selecting obvious mutant areas, measuring the entire area, recording cell dimensions and number of recent cross-walls. Visibly normal areas immediately adjacent to the warts were similarly analyzed. Comparable positions on non-mutant leaves, as measured from tip to emerging ligule, were selected and similarly analyzed for cell dimensions and cross-wall number/area. The quantitative analysis displays the results of the means of three individuals of mutant and non-mutant siblings, as plotted in Figs 8-10.

RESULTS

During normal development, leaf primordia emerge from, and encircle, the base of the shoot apical meristem, forming a crescent shaped buttress, which eventually grows up and around the meristem to produce the typical strap-shaped grass leaf (Fig. 1). Initially, the emerging leaf buttress (Fig. 1A) forms a hooded primordium (Fig. 1B, top), which is broad based, almost heart-shaped with a recurved tip (Fig. 1B, bottom). Next, the basal margins of the leaf blade expand laterally and the tip extends up to form a cone-shaped leaf (Fig. 1C, top), which is spade-shaped in adaxial view (Fig. 1C, bottom). The blade grows upward first, followed later by the sheath, producing the typical linear shape of the maize leaf (Fig. 1D). A forming ligule separates the upper blade from the lower sheath (line parallel to leaf base in Fig. 1C, bottom). The base of the blade immediately above the ligule retains the outward curvature of the hood and cone-shaped primordium, but the relative width to length of the blade decreases considerably as the leaf acquires its final strap-shape. Cell axiality is maintained throughout development. Any developmental alteration in division or expansion, which occurred early, is thus detectable later in the mature leaf. Mature leaves were thus screened for cell shape abnormalities and developmental analysis was conducted on the stages depicted in Fig. 1C.

Wty-1 mutants and non-mutants grow at similar rates

The *wty-1* allele segregates as a recessive mutation, with mutant and non-mutant plants phenotypically similar in overall appearance and flowering time (Fig. 2A). Adult mutant and non-mutant leaves appear identical in size and shape, except that fully mature leaf blades have a variable and sporadically bumpy epidermal surface. The bumps or warts, where present, are linearly aligned in patches, giving the leaf a corrugated and slightly gritty texture (Fig. 2B). *wty-1*, *bum-1* and *rl-1* are allelic, based on crosses between mutants homozygous for each allele (data not shown).

Proportions of leaf regions were compared in mutant and non-mutant individuals by calculating blade to sheath ratios of developing seedlings (Fig. 3). Proportions of leaf regions remain similar both during development and once the leaf has

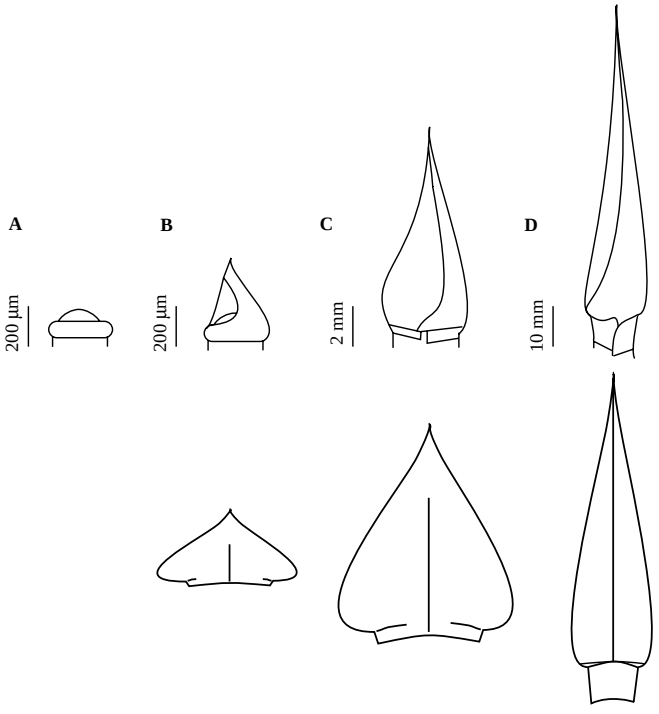

Fig. 1. Morphogenesis of maize leaves. (A) Leaves encircle the shoot apical meristem, then grow up to form a hooded primordium (B, top), a cone-shaped leaf (C, top) and eventually the typical strap-shaped maize leaf (D, top). (B,C,D bottom) adaxial view of isolated leaves shows the comparable change in shape that occurs at each stage.

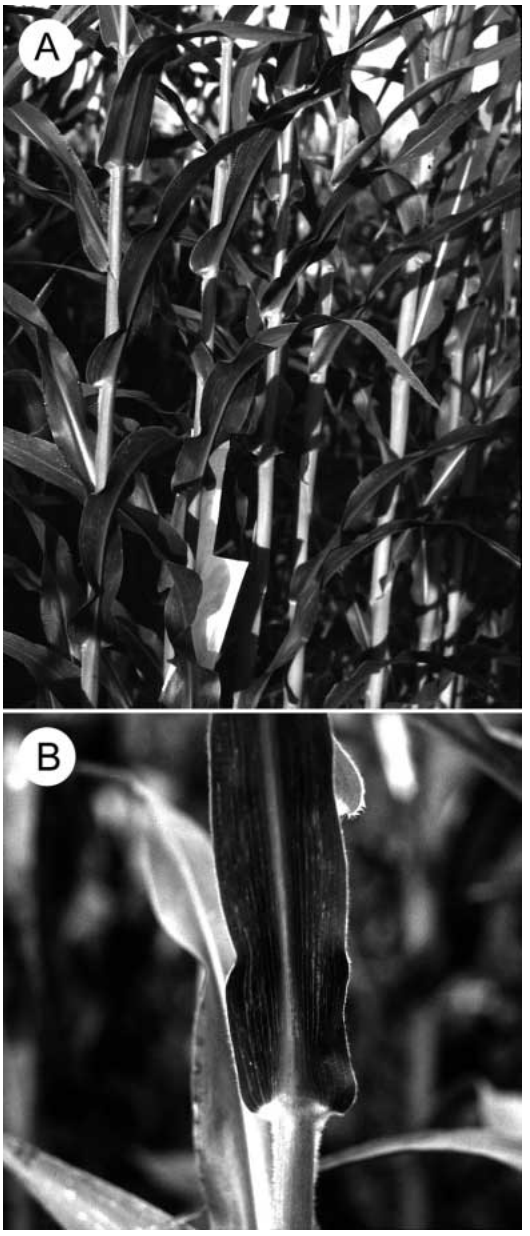

Fig. 2. *Wty* mutant phenotype. (A) *Wty-1* mutants are identical to non-mutant siblings in terms of general appearance and flowering time. (B) Leaf blades of mature mutant plants have a bumpy texture, with apparent warts appearing like streaks in the leaf surface.

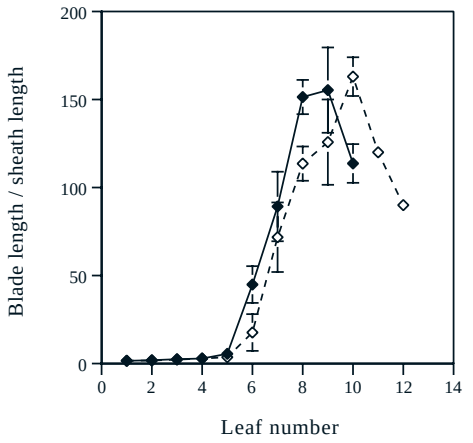

Fig. 3. Proportions of blade and sheath in growing mutant and non-mutants. Blade to sheath ratios are similar in sequential leaves of both mutant (open diamonds) and non-mutant (closed diamonds), suggesting the mutation does not affect leaf proportions or growth rate.

matured. Mature juvenile leaves (leaves 1-4; Fig. 3) reach the same blade to sheath proportions in mutant as in the non-mutant individuals. During juvenile leaf maturation, blade expansion precedes sheath expansion in the same numbered leaf in both mutant and non-mutant (leaves 4-7; Fig. 3). Immature adult leaves of the same leaf number also have similar proportions of blade to sheath in mutant and non-mutant (leaves 8-12; Fig. 3), suggesting that both mutant and non-mutant generate similarly proportioned leaves. Both mutant and non-mutants also produce tassels, shed pollen and develop ears at the same time (data not shown).

Warts in mature mutant adult leaves consist of abnormally expanded cells

The mature mutant leaf blade phenotype was examined in cleared and stained leaves (Fig. 4A,B). Warts are restricted to the blade of adult leaves and consist of overly expanded cells in both the adaxial and abaxial epidermis. Linear axiality of mutant cells is generally maintained, but warty cells are enlarged and some are improperly positioned (arrowhead in Fig. 4B points to a misplaced bullliform cell in the midst of an intercostal cell row, as indicated by the purple stain). In cross-section, epidermal cells appear slightly enlarged (compare Figs. 4C,D). Warts are visible in both the adaxial and abaxial epidermis as excessively enlarged cells (Fig. 4C,D). However, overlying regions of each epidermis are independently over-expanded because enlarged cells in the adaxial epidermis do not correspond with warts on the abaxial side. Cell sizes are also abnormal in internal leaf tissues including vascular tissues and leaf mesophyll cells (Fig. 4D). Cell enlargement is restricted to the leaf blade; other regions, including sheath, ligular region, as well as non-leaf and reproductive organs do not show the cell enlargement phenotype (data not shown).

Developing mutant leaves show abnormal cell division patterns and overly expanded cells

Immature 1 cm long adult leaves were examined for the first signs of the mutant defect. Recently divided cells can be recognized by the thin cell plates visible in nail polish casts of leaf impressions when viewed with dark-field optics (Fig. 5A,B). In non-mutant leaves, new cell walls are complete and follow the axial relationship to the local leaf axis (Fig. 5A; all micrographs are aligned with the local longitudinal axis parallel to the page). Recent cell pairs contain either longitudinally oriented new walls (arrow, Fig. 5A) or transversely oriented new walls (arrowheads Fig. 5A). Mutant cells, however, have many incomplete and abnormally positioned new walls (arrowhead, Fig. 5B). In transverse section, however, mutant and non-mutant leaves appear identical (Fig. 5C,D). Abnormalities of cell expansion and of cell plate deposition are thus only detected in surface view of epidermal cells at this early developmental stage of the leaf.

The developing leaf is divided into a pre-differentiation and a differentiation zone, corresponding with changing dimensions of dividing cells and with the start of cell morphogenesis

Most of the shape changes of the leaf occur during the hood and cone-shaped stage, as depicted in Fig. 1A-C (compare top and bottom illustrations). Remarkably, cell axiality is maintained during these subtle changes in leaf shape. Linear files of cells are aligned locally with the axis of a given leaf region. Cell size, as a function of division and expansion, were compared in mutant and non-mutant sibling leaves. Cone-shaped leaves (stage comparable to Fig. 1C) have a distinct zonation of cell sizes between the emerging ligule region and the leaf tip, as stylized in Fig. 6. The zonation reflects gradients of cell size that can be attributed to changing patterns of division, expansion and differentiation. As described for maize (Sylvester et al., 1990; Poethig and Szymkowiak, 1995) division and expansion occur throughout the cone-shaped leaf but in opposing patterns. Progressing from base to tip, new cross-walls appear to decrease in number while cell sizes increase. Cell differentiation begins about half way up the 1 cm leaf in the ND101 background. Differentiation is recognized by cell morphogenesis at a restricted border; that is cells begin to acquire unique shapes reflecting their future function. This differentiation border correlates with the position where cells have achieved a predictable volume prior to the onset of cell morphogenesis. The differentiation border of the epidermis is surprisingly narrow and is defined here as a generalized position, not a defined boundary, where cell morphogenesis is first recognized. The differentiation border is not necessarily the position where cell fates are determined, but is a visibly convenient marker for the start of cell morphogenesis. In maturing strap-shaped leaves, the differentiation border is still narrow and starts 2-4 cm above the fully established ligule (data not shown).

Cells in the pre-differentiation zone were examined and used for quantitative analysis. The emerging ligule region can be detected by the small cell size and outward growth of preligular cells as previously described (Sylvester et al., 1990). The pre-differentiation zone above the ligule is divided into sub-zones of increasingly longer cells, as shown for a non-mutant individual in Fig. 7A-D. The basal most sub-zone, just above the ligular band, consists of squarish cells two to three times as long as wide (Fig. 7D), similar to ‘tiles’ in appearance. Above this zone are cells approx. four times as long as wide, similar to ‘bricks’ because of their more elongate axis (Fig. 7C). These bricks continue expanding anistropically, producing the third upper sub-zone of ‘long bricks’ (Fig. 7B),

until the differentiation border is reached (Fig. 7A). For convenience, the three sub-zones are named here, based on position within the pre-differentiaton zone and thus are called basal, mid and upper sub-zones. Hairs start to emerge at the border of the differentiation zone (arrowhead, Fig. 7A,E). The remainder of the leaf is the differentiation zone in which macrohairs, bulliform cells and distinct cell features such as crenulated walls are observed (data not shown). Above the differentiation zone in strap-shaped leaves is the maturation zone, where differentiating cells acquire their mature size and function. This maturation zone is not visible in the cone-shaped leaves and so is not discussed further here.

The mutant defect is first visible in the upper sub-zone of the pre-differention zone, but is not detectable in the basal or mid sub-zones

Mutant leaves show the same distribution of cell sizes in the pre-differentiation zone as observed in non-mutants (compare Fig. 7D to H, 7C to G and 7B to F). Mutant cells in the basal and mid sub-zones look identical to non-mutant (Fig. 7C-H). Enlarged cells (warts) are first visible in the upper sub-zone (arrow Fig. 7F), where abnormally positioned and incomplete cross-walls are also observed. At the differentiation border, enlarged mutant cells are more pronounced; the cells are relatively larger compared with adjoining cells and the warts are more frequent. These observations were consistent in all pre-differentiation zones examined, including the pre-differentiation zones of strap-shaped developing leaves (data not shown).

Two observations suggested that the mutation will be useful for understanding the relationship between cell division and cell expansion. First, the mutant defect is first visible in a restricted sub-zone of the leaf that correlates with a predictable cell size. Second, it is clear that cytokinesis and/or mitosis is affected in these same enlarged cells. Division in relation to cell size was quantified to determine if altered cytokinesis preceded or coincided with the change in cell size. Figure 8 shows length as a function of width for classes of division orientation (basal sub-zone, Fig. 8A; mid sub-zone, Fig. 8B; upper sub-zone, Fig. 8C). The results show that cells of similar dimensions divide at particular orientations, regardless of location. Long thin cells tend to contain recent transverse cross-walls, whereas short broad cells tend to contain recent longitudinal cross walls in the basal sub-zone for mutants and non-mutants (Fig. 8A). Cells in the mid sub-zone are overall longer and thinner, but division orientations still correlate with cell size (Fig. 8B). Surprisingly, a class of cells, enlarged by 133%, is present in the mutant. These enlarged cells contain complete and normal cross-walls, all of longitudinal orientation. Thus, although the mutation is clearly affecting cell size at this position, there is not yet a visible effect of the mutation on the orientation or execution of cytokinesis.

The mutation does not affect cytokinesis until cells exceed a certain size ratio, shown in the upper sub-zone. These cells, or warts, are abnormally enlarged and all have abnormal or incomplete cross-walls. Quantification of cell sizes shows that the majority of mutant and non-mutant cells of a given division class are of similar size (Fig. 8C). However, a distinct class of mutant cells in the warts are exceptionally enlarged (Fig. 8C). These cells average a 200% increase both in cell length and width compared with non-mutant cells. Also, the enlarged

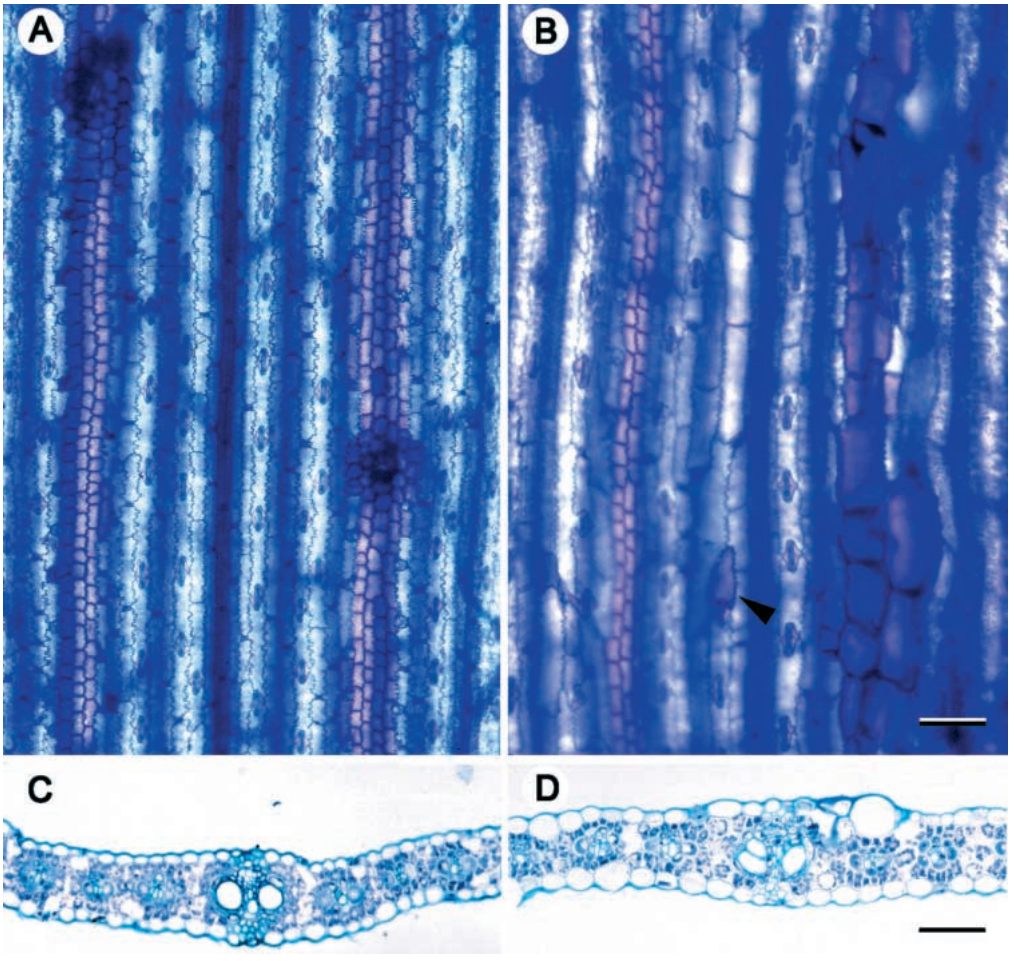

Fig. 4. Phenotype of the mature adult leaf in non-mutant (A,C) and mutant (B,D). (A) Cells are orderly and of predictable sizes in non-mutant compared with (B) the overly-expanded cells characteristic of a wart in a mutant adult blade. Purple stain indicates bulliform cells and subsidiary cells. Note the misplaced purple-stained cell in the midst of a wart (arrowhead, B). (D) Adaxial and abaxial mutant epidermis, as well as cells in the mesophyll and vascular bundles, are overly expanded in the mutant compared with non-mutant (C). Scale bars, 70 μm (A,B); 186 μm (C,D).

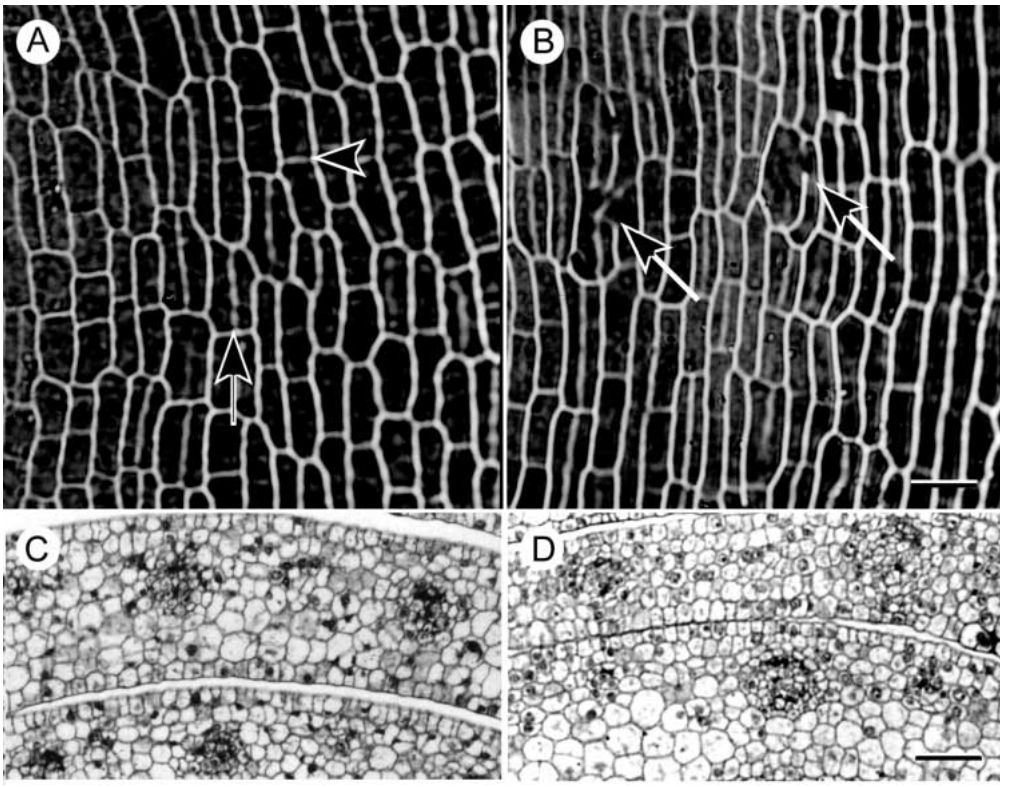

Fig. 5. Phenotype of immature adult leaf in non-mutant (A,C) and mutant (B,D). Transverse (arrowhead, A) and longitudinal divisions (arrow, A) are abundant and axial in orientation in non-mutant. In addition to these classes, developing mutant leaves show clusters of cells with abnormal and incomplete cross walls (arrows, B). There is no difference in cell size or apparent division at this stage between non-mutant (C) and mutant (D). Scale bars, 35 μm (A,B); 118 μm (C,D).

mutant cells contain abnormally positioned or incomplete longitudinal cross-walls.

The results show that, despite the first appearance of enlarged cells in the mid sub-zone (Fig. 8B), abnormal cytokinesis does not occur until cells exceed a predictable size ratio in the upper sub-zone (Fig. 8C). To examine this further, size ratios were calculated (cell length divided by cell width) and these data were compared for mutant and non-mutant (Fig. 9). In non-mutants, as expected, cells with recent transverse divisions have a size ratio of about 7, whereas cells with recent longitudinal division have a size ratio of about 3. In the mid sub-zone, where enlarged cells still have normal division patterns, the size ratio of the enlarged cells remains typical for normal-sized mutant cells. Thus, length and width increases equally and division orientations are still normal. In the upper sub-zone, enlarged cells that exceed a size ratio characteristic for normal longitudinal divisions show consistently altered division patterns. The trends depicted in Fig. 9 suggest that cells of atypical size ratios divide abnormally, and that these atypical ratios are reached in cells of a given age, size and position within the developing mutant leaf.

Potential compensation between division and expansion in the differentiation zone

The mutant phenotype is first *visible* in the upper sub-zone (Fig. 7F) as a developing wart, but is first *measurable* below in the mid sub-zone (Figs 7G and 8B). The select abnormal cells typical of the wart continue to over-expand (Fig. 7E) up into the differentiation zone. Cell features were further quantified in the differentiation zone to test for the possibility that cells adjacent to the developing wart are also affected, either by the mutation itself or as compensation for the over-expansion experienced by cells in the wart.

In the differentiation zone of 1 cm mutant leaves, visible warts are adjacent to cells that appear normal in other respects. These two adjoining fields of cells (‘abnormal’ mutant cells next to ‘normal’ mutant cells) were selected for quantitative comparison. Size ratios were first calculated for the adjacent ‘abnormal’ and ‘normal’ cells in the mutant and for comparable zones in the non-mutant sibling. First, as expected,

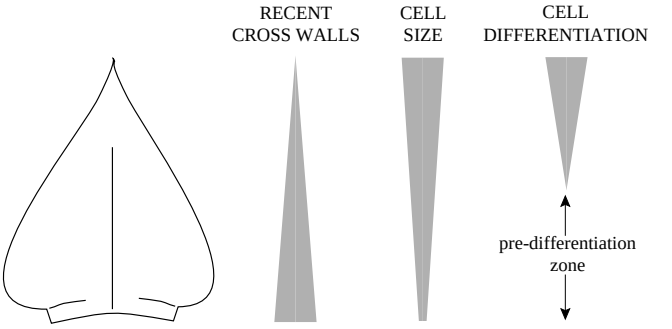

Fig. 6. Gradient of new cross-walls, cell sizes and differentiation in a 1 cm cone-shaped maize leaf (ND-101). In adaxial view, a 1 cm leaf is spade-shaped with distinct vasculature (the midvein only is depicted as the central vertical line) and a forming ligule (depicted as a basal horizontal line). Cell division and cell expansion are inversely related: division slows while cell expansion increases in acropetal direction. Divisions are either transverse or longitudinal. Cell differentiation starts about half-way up the leaf and is recognized by the first emergence of hairs. The pre-differentiation zone, below the border where differentiation starts, shows a gradient of cell sizes. The pre-differentiation zone is classified arbitrarily into sub-zones based on cell shapes (see Fig. 8).

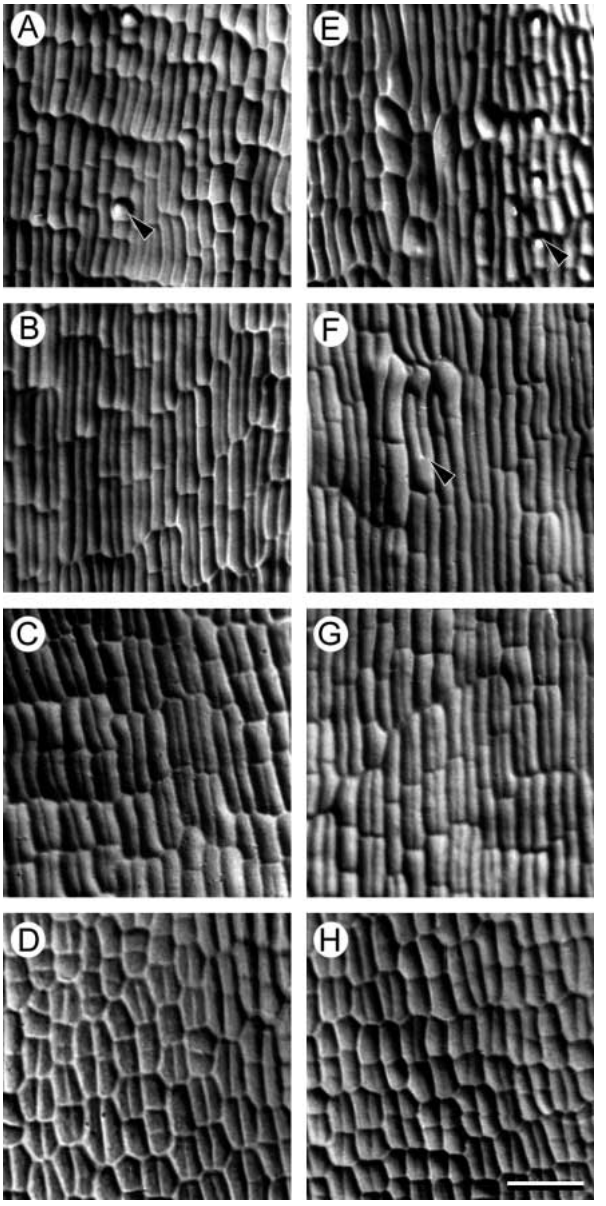

Fig. 7. Distribution of cells in sub-zones in a 1 cm cone-shaped leaf of non-mutant (A-D) and mutant (E-H). The differentiation border starts halfway up a 1 cm leaf of ND101 background in both non-mutant (A) and mutant (E) and is recognizable by emergent hairs (arrowheads, A,E). For convenience, sub-zones are basal (D,H), mid (C,G) and upper (B,F). Warts are first visible in the upper sub-zone, where overly expanded cells with abnormal cross-walls are seen (arrowhead, F). The warts are even more apparent at the differentiation border (E). Scale bar 110 μm.

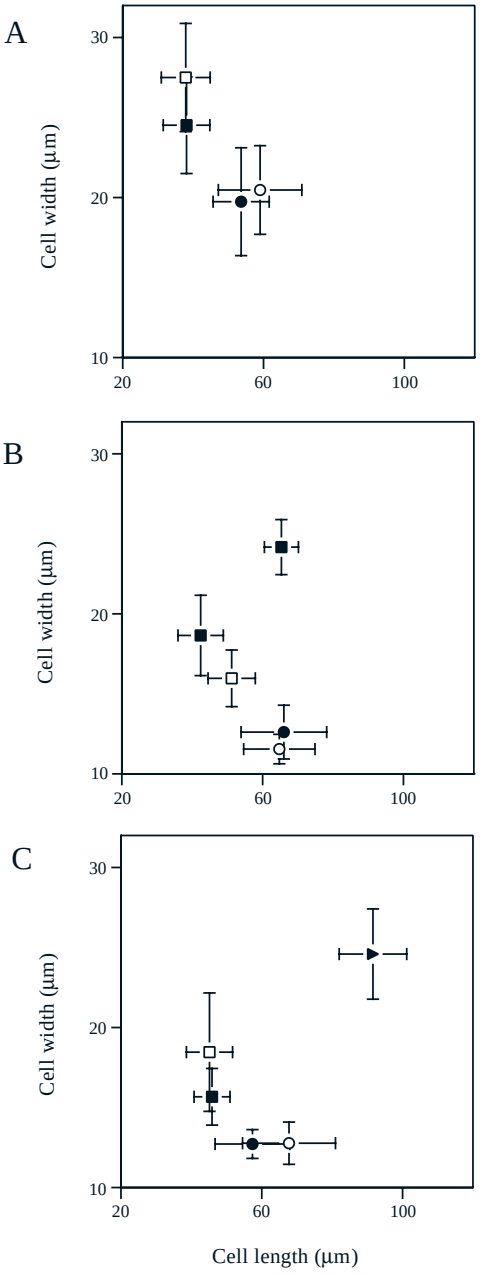

Fig. 8. Cell dimensions in relation to division orientation in (A) basal, (B) mid and (C) upper sub-zones. Cell dimensions are classified according to whether the division orientation of cell pairs is longitudinal (squares), abnormal longitudinal (triangles) or transverse (circles). Mutant cells (filled symbols) are compared with non-mutant cells (open symbols). Cells that undergo longitudinal cell divsions in all three sub-zones are shorter and wider than cells that undergo transverse cell division. There is no difference in cell size or division orientation between mutant and non-mutant in basal sub-zone (A; comparable in position to Fig. 7D and H). In the mid sub-zone (comparable in position to Fig. 7C and G), mutant cells that undergo longitudinal divisions fall into two classes: cells that are similar to non-mutant and cells that are longer and wider than normal (B). These enlarged cells have normal cell division orientations. The unique class of cells is also present in the long brick-shaped cells of the upper sub-zone (comparable in position to Fig. 7B and F). These unusually long and wide cells also undergo abnormal and incomplete longitudinal cell divisions.

the abnormal cells in the mutant warts are exceptionally long and wide (Fig. 10A). Surprisingly, normal cells in the mutant (filled circle, Fig. 10A) are considerably shorter overall than the abnormal mutant cells (compare the filled circle with the filled square in Fig. 10A) and are also smaller than non-mutant cells (open diamond, Fig. 10A).

Recent cross walls were measured in the same adjoining regions of normal and abnormal mutant cells in the differentiation zone, to determine the basis for the smaller

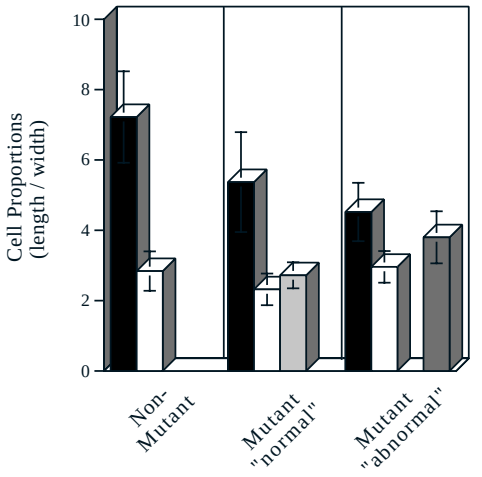

Fig. 9. Cell size ratios in non-mutant and mutants of the middle sub-zone compared with non-mutants of the upper sub-zone. Size ratios are calculated for cells containing transverse divisions (black bars) longitudinal divisions (white bars), normal longitudindal division but in enlarged cells (lightly shaded bars) and abnormal longitudinal division in enlarged cells (heavily shaded bars). Normal longitudinal divisions in enlarged cells in mutants are within a size range characteristic of non-mutant. Cells exceeding normal ranges of size ratios also divide abnormally.

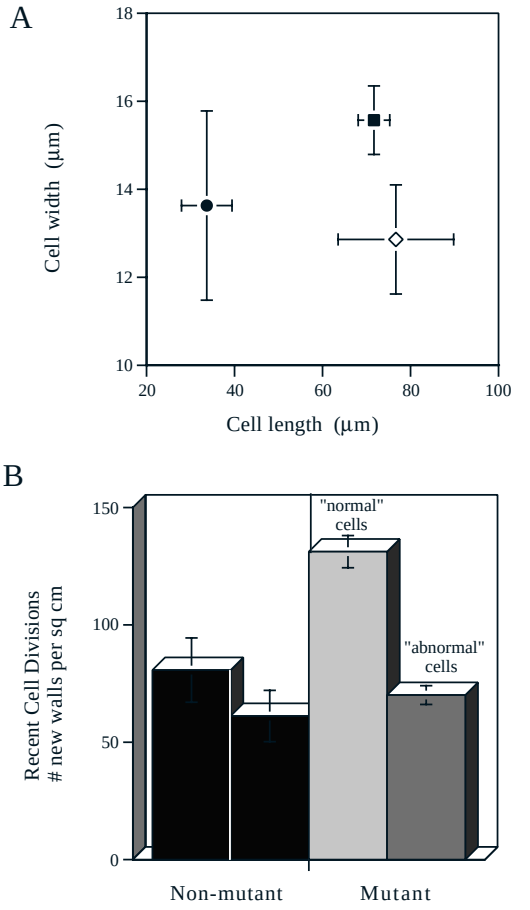

Fig. 10. Cell dimensions and division in the differentiation zone. Cell sizes are compared in adjoining regions of mutant in which abnormal and enlarged cells (A; filled squares) are adjacent to normal looking cells (A; filled circles). These were compared with cells in a comparable position in non-mutant (A; open diamond). 'Abnormal' mutant cells are longer than non-mutant; but 'normal' mutant cells are shorter than either non-mutant or the 'abnormal' mutant cells. In B, non-mutant cross walls (dark shaded bars) are compared with an 'abnormal' mutant (medium-shaded bars) with an adjoining 'normal' area of mutant. 'Normal' cells that adjoin the enlarged 'abnormal' mutant cells are dividing faster and are smaller than non-mutant.

cell size (Fig. 10B). The number of recent cross-walls per cm^2 area was used as a measure of cytokinesis. Two adjoining non-mutant regions for the same position along the leaf axis were compared for three individuals, and these were compared with adjoining 'normal' and 'abnormal' regions of three mutant individuals. The abnormal cells in the mutant have similar numbers of recent cross-walls as the non-mutant, despite the fact that the cells are exceptionally larger. In contrast, the adjoining normal region of the mutant has significantly more cross-walls than either non-mutant or the abnormal region of the mutant. Combined, the results suggest that normal mutant and abnormal mutant cells that adjoin are opposite in their patterns of cell division and expansion: abnormal mutant cells are dividing less and enlarging considerably, whereas normal mutant cells are dividing more and are enlarging less.

DISCUSSION

Wty-1 is a mutation that affects expansion and division of cells in the maize leaf without altering leaf shapes, flowering or other aspects of plant growth. To analyze cell pattern, a developing 1 cm leaf is subdivided into a pre-differentiation and a differentiation zone, which is distinguished by cell morphogenesis. The pre-differentiation zone is further divided into sub-zones based on cell dimensions. First, we characterize a predictable progression of cells sizes in the pre-differentiation zone during normal development. Cells in the pre-differentiation zone are oriented with the local leaf axis, and progress in a predictable size gradient that spans approximately 500 µm up the leaf from the developing ligule. Cell shapes correspond to the size ratio of the cells. Furthermore, the orientation of division plane correlates with cell size ratios in all three sub-zones examined. Thus, division orientation correlates with degree and extent of cell expansion in the normal condition.

Next, we contrast the normal size changes in the pre-differentiation zone of mutant and non-mutant siblings. The mutant defect is first visible in select cells that exceed a given size ratio; these cells continue to enlarge and also to divide abnormally. The intriguing finding is that cytokinesis does not appear abnormal until a particular size threshold is surpassed in these cells; that is, some mutant cells in the mid sub-zone are measurably enlarged, but still have normal longitudinal cross-walls. These cells average only 133% increase in length and width compared with the non-mutant. Cytokinesis is abnormal in the upper sub-zone, a position where cells exceed a 200% increase in size.

The results show that cytokinesis is not visibly altered until size ratios are abnormal. The events that generate the altered size ratio have not been analyzed and indeed cannot be

distinguished because rates of division and expansion of individual cells are not evaluated here. Expansion rate could be initially elevated in pre-wart cells, thus producing cells that more quickly exceed a size ratio than that characteristic of neighboring non-wart cells. Alternatively, division rate could be depressed, or transverse divisions could be absent, producing a similar result. Regardless of how warts are initially generated between the basal and mid sub-zones, cytokinesis does not become visibly aberrant until cells surpass the recognized size threshold.

These results suggest that new cross-walls are abnormal in cells that are between one and a half and two times the normal size. One possibility is that the cell plate is mechanically unable to reach the greater distance required by such a large cell. It is interesting to note that the abnormal cross-walls in the cells that have exceeded the threshold are all close to longitudinal, the longest distance a forming cell plate would have to travel during cytokinesis. Other hypotheses could explain the results, including: a biochemical imbalance in the cells, thus preventing proper signalling for cytokinesis; altered cell wall properties that prevent normal controlled expansion; improper cytoskeletal function that prevents signalling by the preprophase band and/or prevents proper guidance of the cell plate by the phragmoplast. Cells in warts could also have an improper nuclear to cytoplastic cell volume, thus precluding normal cytokinesis. At this level of analysis, it is not possibile to determine the nature of the mutation, except we note the fact that cell volumes must exceed a threshold before cytokinesis appears to be altered.

Several issues can be considered using the *WTY* gene. One issue has to do with how cell division and expansion interact and feed back on one another during growth and development. Are the two processes interdependent? And to what extent does the direction and rate of one regulate the extent and direction of the other? Our results suggest that enlargement of cells in the *wty-1* mutant precedes aberrant cytokinesis, suggesting that some aspects of cytokinesis are dependent on proper cell size. Consistent size ratios of cells may be required for proper direction and completion of cytokinesis. It is also important to consider other cellular events occurring in growing cells. For example, the division plane in vacuolated cells is influenced by the distribution of cytoplasm (Sinnot and Bloch, 1940; Lloyd, 1991), which is more variably distributed in enlarged cells of the upper sub-zone than in the compact cells of the basal sub-zone. Thus, altered division in the mutant could be indirectly due to improper disposition of cytoplasm in the large increasingly more vacuolate cells.

Speculation as to the nature of the *WTY* gene product will not be meaningful until genetic and molecular analysis is complete. It is likely that *wty-1*, the subject of this investigation, is a null mutation because two other recessive alleles (*bum-1* and *rl-1*) show identical phenotypes, but such speculation awaits cloning the gene and further characterization of the mutant alleles. There are other unexplained features of the mutant phenotype, particularly concerning the distribution of warts: for example, the patchy nature of the warts and the localized distribution in blade only are both puzzling features. Restriction to blade tissue only could be explained by the fact that formation of the blade requires shape changes not encountered in other more uniformly shaped organs; that is, roots, culms and leaf sheaths are all more-or-less radially symmetrical, whereas the blade shows a unique flattened bilateral symmetry that may require more complex changes in cell polarity during morphogenesis. Additionally, blade cells may achieve dimensions not present in other organs.

The question of the patchy distribution of warts is also puzzling. It is not yet clear whether warts are stochastic or non-randomly distributed in the blade. We show here that adjoining abnormal cells in the warts are not similar to the normal mutant cells in terms of division rate and cell size. It is possible that such opposing cell pattern is due to cross-talk among adjoining mutant cells such that the normal-appearing mutant cells divide more to compensate for the overly expanded warty cells. This first model argues that the mutation only affects some cells directly and that neighboring cells respond to the mutant defect by altering the normal cell pattern. Alternatively, patchiness could be explained by a generalized de-regulation of the cell cycle, with some cells cycling faster, while others cycle slower. This second model argues that all cells are affected, but that the regulation of cycling varies among cells. The models can be tested when genetic and molecular analysis of the *wty* alleles is completed.

Another issue pertinent to the *WTY* gene has to do with how cells communicate to generate normal leaf shapes. Compensation among cells to maintain organ integrity has long been recognized as fundamental to plant development. Green (1994) suggests that fields of cells behave as units, guided by physical principles of any mechanical system. For example, groups of cells orient and grow in directions corresponding to growth changes within an organ. Coordinated behavior among cells has been seen on the level of cell wall microfibril alignment (Green and Lang, 1981), as well as in the organization of cortical microtubule arrays (Selker and Green, 1984; Marc and Hackett, 1989; Sylvester et al., 1989) and preprophase bands (Jesuthesan and Green, 1989). Freeling (1992) observed that sectored plants, in which slow and fast growing tissues adjoin, are able to adjust to one another to maintain relatively normal leaf widths. All these results support the hypothesis that adjoining cells could regulate cell cycle rates in order to compensate for local disturbances, such as experienced in the *wty* mutant.

The cell cycle in plants includes cell division and expansion and both are intimately associated with the microtubule cycle (Mineyuki et al., 1991; Traas et al., 1992; Colasanti et al., 1993; Cyr and Palevitz, 1995). A long-standing question has been how the microtubule arrays themselves may signal the direction of cytokinesis and cell expansion (for reviews see Lloyd, 1991; Cyr and Palevitz, 1995). Single cell systems reveal precise roles of the microtubule cytoskeleton in maintaining many aspects of polarity (Roemer et al., 1996; Mata and Nurse, 1997). In yeast cells, for example, the *tea1* gene is required to supply necessary spatial information for maintaining cell polarity and the microtubule cytoskeleton localizes *tea1* at the proper position (Mata and Nurse, 1997). In plants, the situation is further complicated by the three-dimensions of a tissue system, but experimental evidence supports the important role of microtubule arrays, such as the preprophase band, in cell division and expansion (Gunning and Wick, 1985; Mineyuki and Gunning, 1990; Lloyd, 1991). It is tempting to speculate that the *wty* mutation will lead to the identification of a gene important in engaging the microtubule cycle with the cell cycle. Preliminary analysis reveals that microtubule arrays are not normal in mutant cells, but future research will help to clarify this issue.

Single cell systems, such as those described for yeast, provide baseline models for understanding morphogenesis in

complex three-dimensional tissues of plants. Other baseline data from analysis of plant root development are also pertinent (Benfey et al., 1993, Baskin et al., 1995, Scheres et al., 1995, Hauser et al., 1995, Scheres et al., 1996). The shoot system, while certainly conserving basic cellular mechanisms, may show complexities of morphogenesis not encountered by the unidirectionally growing root system. Primarily the alleles of *wty-1* (this paper), *tan-1* (Smith et al., 1996), *crinkly-4* (*cr4*; Becraft et al., 1996) have been identified as relevant to the issues of cell growth and leaf morphogenesis. Further identification of additional loci will contribute to our understanding of how morphogenesis is controlled.

The sub-zones of the pre-differentiation zone described here are present in both non-mutant and mutant individuals. Division (orientation and rate) must be delicately balanced with expansion (orientation and rate) to produce the predictable cell sizes observed. The *wty-1* mutant reveals that a remarkable degree of abnormal cell enlargement can be tolerated with little influence on large-scale morphogenesis. Furthermore, once the balance is offset two events can occur. First, cell cycles in adjoining cells are altered and second, overly expanded cells lose the ability to divide normally. The mechanism controlling these local interactions will become clearer as additional mutants are found, genes cloned and their products analyzed. Understanding what regulates the balance between division and expansion will be critical to clarifying what drives morphogenesis.

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