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ORIGINAL ARTICLE

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Stomatal neighbor cell polarity and division in *Arabidopsis*

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Abstract Asymmetric divisions are key to regulating the number and patterning of stomata in *Arabidopsis thaliana* (L.) Heynh. Many formative asymmetric divisions take place in neighbor cells (NCs), cells adjacent to a stoma or stomatal precursor. *TOO MANY MOUTHS* is a receptor-like protein required for the correct plane of NC division, resulting in the placement of the new precursor distal to the pre-existing stoma. Because plant cells usually become polarized before asymmetric division, we studied whether NCs display a cytological asymmetry as a function of cell stage and of possible division behavior. Cells that divided in the developing leaf epidermis were smaller than 400 μm^{-2} in area and included NCs as well as isolated cells. All NCs in the youngest complexes divided with comparable frequencies, but divisions became restricted to the smaller and most recently produced NCs as the stomatal complex matured. The majority of developing NCs had distally located nuclei, suggesting that nuclear position is actively regulated in NCs. NC stages exhibiting distally located nuclei were the likeliest to divide asymmetrically. However, a distal nucleus did not necessarily predict an asymmetric division, because more NCs had distal nuclei than were likely to divide. No defect was detected in nuclear distribution in *tmm* NCs. These data suggest that *TMM* uses intercellular signals to control the plane of asymmetric division after or independently of nuclear positioning.

Keywords *Arabidopsis* · Asymmetric division · Polarity · *TOO MANY MOUTHS*

Dedicated to the memory of Judith Croxdale for her contributions to the study of stomatal patterning.

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Abbreviations GFP: green fluorescent protein · GMC: guard mother cell · MMC: meristemoid mother cell · NC: neighbor cell · *TMM*: *TOO MANY MOUTHS* · WT: wild type

Introduction

Arabidopsis stomata are produced and patterned via asymmetric divisions (von Groll and Altmann 2001; Nadeau and Sack 2002a). The first asymmetric division in this pathway produces a meristemoid, a precursor cell that eventually forms the stoma (Fig. 1). Stomatal number is thus proportional to the frequency of formative asymmetric divisions (Geisler and Sack 2002). Cells undergoing these and other divisions can be considered stem cells that act during the post-protodermal phase of leaf development (Nadeau and Sack 2002b).

Stomata are spaced apart from each other by at least one cell (Larkin et al. 1997; Geisler et al. 1998). We define a neighbor cell (NC) as one that is adjacent to a stoma, a meristemoid or guard mother cell (GMC). Asymmetric divisions in NCs pattern the majority of stomata in *Arabidopsis* leaves (Geisler et al. 2000). The plane of this division is oriented so that the new satellite meristemoid is placed away from (distal to) the pre-existing stoma or precursor (Fig. 1; Geisler et al. 2000).

NCs probably receive positional cues via cell–cell signaling that are used to orient the plane of division. Mutations in the *TOO MANY MOUTHS* gene randomize the orientation of these divisions and create pattern violations, i.e. clusters of adjacent stomata (Fig. 1c; Yang and Sack 1995; Geisler et al. 2000). *TMM* is a leucine-rich repeat receptor-like protein that is expressed in NCs as well as in stomatal precursors (Nadeau and Sack 2002b). These data support the hypothesis that TMM receives or interprets extracellular spatial signals to correctly place the site of asymmetric division.

Prior to asymmetric division, animal and plant cells often become polarized with respect to the distribution of organelles or of specific proteins, such as cell fate

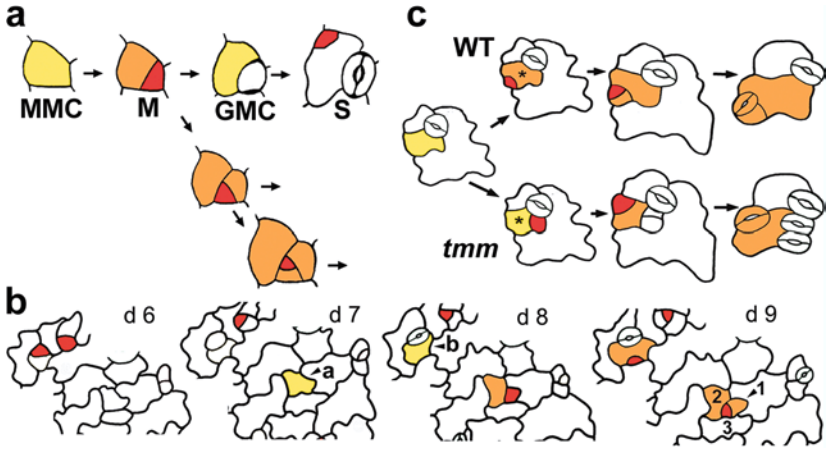

Fig. 1a–c Stomatal development in *Arabidopsis thaliana*. *Yellow* Meristemoid mother cell (*MMC*), *red*, meristemoid (*M*), *tan* derivatives of selected MMCs. **a** The first asymmetric division produces a meristemoid. Meristemoids can divide before converting into a guard mother cell (*GMC*) that forms the stoma (*S*). **b** Dental resin series from a WT cotyledon over a 4-day period (days 6–9). MMC "*b*" (day 8) is an NC that divides asymmetrically producing a correctly patterned satellite meristemoid by day 9 (days after germination). MMC "*a*" (day 7) is not an NC. Two successive asymmetric divisions produce NCs *1* and *2* by day 9. Adapted from Geisler et al. (2000). **c** Dental resin series from *tmm* (*bottom*) illustrating ectopic placement of satellite meristemoid. Cartoon of probable WT sequence (*top*). Cells (*) next to two stomata and/or precursors normally do not divide but do in *tmm*. Adapted from Nadeau and Sack (2002b)

determinants (Scheres and Benfey 1999; Knoblich 2001). Before the division of monocot subsidiary mother cells and *Arabidopsis* meristemoids, the nucleus migrates to one pole of the cell and the vacuole occupies the other (Fig. 2a–c; Galatis and Mitrakos 1979; Kennard and Cleary 1997; Zhao and Sack 1999). A preprophase band of microtubules usually surrounds the polarly located nucleus (Smith 2001). This band marks the division site, the location where the cell plate later fuses with the parent cell wall (Mineyuki 1999).

To define further the role of TMM in the spatial control of NC division, we studied whether nuclear migration occurs in NCs, whether cell polarization predicts an asymmetric division, and whether these events are disrupted by *tmm*. We also analyzed whether the probability of asymmetric division in an NC is related to its age, size, and to its adjacent cell type (stoma or precursor). Here we report that classes of NCs likeliest to divide showed the highest frequencies of distally polarized nuclei. But more NCs were polarized than were predicted to divide, and *tmm* does not appear to disrupt nuclear position.

Materials and methods

Plant material

Plants used for electron microscopy were grown and processed as in Zhao and Sack (1999). All other plants were grown aseptically in plastic Petri dishes on 0.8% agar containing 1% (w/v) sucrose and minerals (Sigma-Aldrich, St. Louis, Mo., USA). Seedlings were

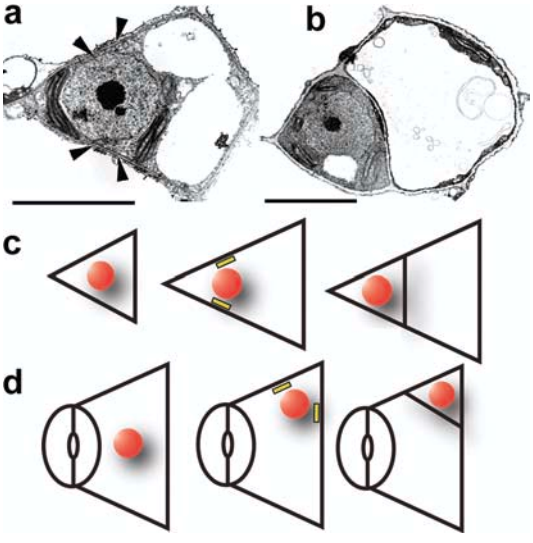

Fig. 2a–d Nuclear position and asymmetric division. **a, b** Adapted from Zhao and Sack (1999). **a** Polarized meristemoid with nucleus at one pole and vacuole at the other. *Arrowheads* Preprophase band of microtubules that indicates division site. **b** Asymmetric division regenerates meristemoid (*left*) and produces larger sister cell. **c** Events during asymmetric division of meristemoid. Non-polarized cell with central nucleus (*left*) develops asymmetrically positioned nucleus and division site (*yellow bars*). **d** Hypothesized comparable sequence for production of satellite meristemoid by NC. Bars = 5 μm (**a, b**)

maintained in a growth chamber (Percival Scientific, Perry, Iowa, USA.) at 21–22 °C, with a 16:8 h light:dark cycle, and an irradiance from fluorescent lamps of 80–90 μmol m^{-2} s^{-1}. All *Arabidopsis thaliana* (L.) Heynh. plants used were of the Columbia ecotype, including those transformed with green fluorescent protein (GFP; see below). Seeds harboring GFP markers were a generous gift of Sean Cutler, David Ehrhardt, and Chris Somerville from the Carnegie Institute of Washington, Stanford, Calif., USA and were introgressed into *tmm* plants. Except for the GFP-introgressed plants, all seeds were homozygous for the *glabrous1* mutation (Larkin et al. 1997). Neither trichomes nor the trichome-less phenotype of *gl1* affect the one-celled stomatal spacing pattern (Geisler et al. 2000). All data are for the abaxial epidermis.

Cell division

The behavior of cells through time was analyzed from successive dental resin impressions of developing first leaves and cotyledons as described in Geisler et al. (2000).

Asymmetric divisions of neighbor cells

Dental resin series were used to quantify the relative frequency of satellite meristemoid initiation as a function of the stage of the stomatal complex (see Fig. 4a, b). Relative NC age was mostly determined directly from dental impressions (NC1 youngest, NC3 oldest). In some cases NCs were scored based on relative size. Both non-clonal and clonal NCs were included in the sample (Geisler et al. 2000). Some complexes were comprised of two or four NCs. The samples included 24, 22, and 64 complexes containing a meristemoid, GMC or stoma, respectively. Complexes were scored by the stage of the stoma/precursor in the dental resin peel before an NC in the complex divided asymmetrically. Only complexes that produced one or more satellite meristemoids were included in this sample (see Fig. 4b). Data from Tables 1 and 2 are based on the behavior of NCs through time. Only NCs adjacent to a single stoma or precursor cell were scored.

Cell size and division

The paradermal area of 82 meristemoid mother cells (MMCs) was measured in the peel of the dental resin series that preceded the asymmetric division. Areas were also measured for 15 cells that divided symmetrically. These samples excluded the asymmetric divisions of meristemoids and the symmetric divisions of GMCs. Both samples included NCs as well as cells not adjacent to any stoma or precursor. Areas were measured digitally from tracings. Peels were taken either 12 or 24 h apart. In replicas, divisions can only be identified when the surface of the epidermis becomes altered (usually indented) at the new cell wall. Thus, the actual size of the cell just prior to division can only be estimated by this method.

Microscopy

Nuclei were visualized using GFP fluorescence from the N7 line of Cutler et al. (2000). Virtually all interphase epidermal cells in line N7 in leaves and cotyledons exhibited bright nuclear fluorescence. Fluorescence and corresponding bright-field or differential interference contrast optics images were archived using an Olympus AX-70 microscope and a Magnafire cooled CCD camera (Optronics Corp., Goleta, Calif., USA). NC cytology was studied using a Nikon PCM 2000 confocal microscope and lines stably transformed with GFP (Q5 vacuolar membrane, and Q8 cell membrane; Cutler et al. 2000). Fluorescence was examined either in whole mounts of living young leaves or from paradermal slices cut with a fine scalpel. The slices included several layers of tissue and displayed a distribution of nuclei comparable to that in whole mounts. Electron microscopy techniques were as in Zhao and Sack (1999).

Scoring nuclear position

Figure 5 is based upon GFP data from 44–76 wild-type (WT) NCs and 40–137 *tmm* NCs for each category, for a total of 531 NCs for each genotype. Developing leaves one to three were used. Wild-type and *tmm* samples included only isolated complexes, meaning ones in which NCs were not adjacent to any stoma or precursor other than that in the center of the complex. For nuclear scoring, NCs were categorized by their relative size and shape rather than their age because the latter requires data from dental resin series.

To score nuclear position, NCs imaged on screen were divided visually into three regions, central, proximal and distal (Fig. 5a). The proximal and distal regions were adjacent to or away from the stoma respectively.

In smaller NCs (Fig. 5a, right), scoring was based primarily on the relative position of the nucleus. For example, the nucleus was considered to be central when it was equidistant from the distal and proximal walls. Similarly, the nucleus was proximal when it was closer to the stoma/precursor than to the distal region.

In medium to large NCs, (Fig. 5a, left), the nucleus was scored as central when its edge was approximately one nuclear radius away from any anticlinal cell wall. If the distance between the edge of the nucleus and the cell wall was less than one nuclear radius, nuclei were then scored as either proximal or distal. Nuclei were scored as proximal if they were next to or closely flanking the stoma or precursor. All other peripheral nuclei were considered to be distal.

The relative lengths and sizes of the proximal and distal zones varied with cell size. The effects of variation in zone size on nuclear distribution were tested for a sub-sample of cells included in Fig. 5. We determined whether the nuclear distributions that were observed differed statistically from those predicted for random distributions based on relative zone areas. Twenty NCs from each category in Fig. 5b–c were used to derive the predicted distributions.

Nuclear diameter was measured from digital images of each cell using Adobe Photoshop and NIH image software. The diameter of each nucleus was the mean of two measurements at right angles to each other. The area of each scoring region for each cell was derived using Photoshop. The mean area of each zone was then determined for the NCs in each category. The distribution of nuclei for the three zones for each category was compared to a random distribution based on zone area using a Chi-square test.

Virtually all *tmm* stomatal complexes had at least one NC in contact with another stoma or precursor cell, i.e. isolated stomatal complexes were rare, unlike the WT. For this reason, only isolated, younger complexes were scored for *tmm*, i.e. those surrounding meristemoids and GMCs.

Stomatal clusters in *tmm*

The degree of stomatal clustering was measured separately in developing and in mature leaves. Developing leaves (second or third leaves from about 14-day-old plants) were used to score the placement of the first satellite meristemoid. Fresh tissue was visualized using differential interference contrast optics (10 micrographs from each of 10 plants). Satellite meristemoids were analyzed as to whether or not they were in contact with the pre-existing stoma or precursor. Some complexes with more than two stomata/precursors were also analyzed, as long as it was possible to deduce which was the "central" stoma/precursor and which was the first-formed satellite meristemoid. These plants were grown under the same conditions as the plants used for scoring nuclear position.

Mature *tmm* first or second leaves were collected from 23-day-old plants. One leaf was sampled from each of 10 plants. Leaves were cut along the length of their mid-veins. Fifteen microscope fields about 35,700 μm^2 in area were digitized from each half leaf. Scoring was based on "stomatal units" which can be either a single normal stoma or a cluster of adjacent stomata (Yang and Sack 1995). Cluster frequency is the number of stomatal units that are clustered as a percentage of all stomatal units in the sample. Wild-type plants rarely display clusters (Geisler et al. 1998).

Results

Size threshold for division

To help identify stem cells in the leaf epidermis, we quantified the size of cells that divided asymmetrically (MMCs, Fig. 1a) or symmetrically (Fig. 3a). MMCs were larger than symmetrically dividing cells, with mean areas of 223 ± 9 μm^2 and 139 ± 17 μm^2 ($\pm$SE), respectively. Only 3% of all divisions ($n = 97$) took place in cells 400 μm^2 or larger (Fig. 3b). Thus smaller cells function as stem cells in the developing abaxial epidermis. These cells are distributed throughout the young epidermis, but as the epidermis matures they become infrequent and mostly located next to stomata (Fig. 3c–d).

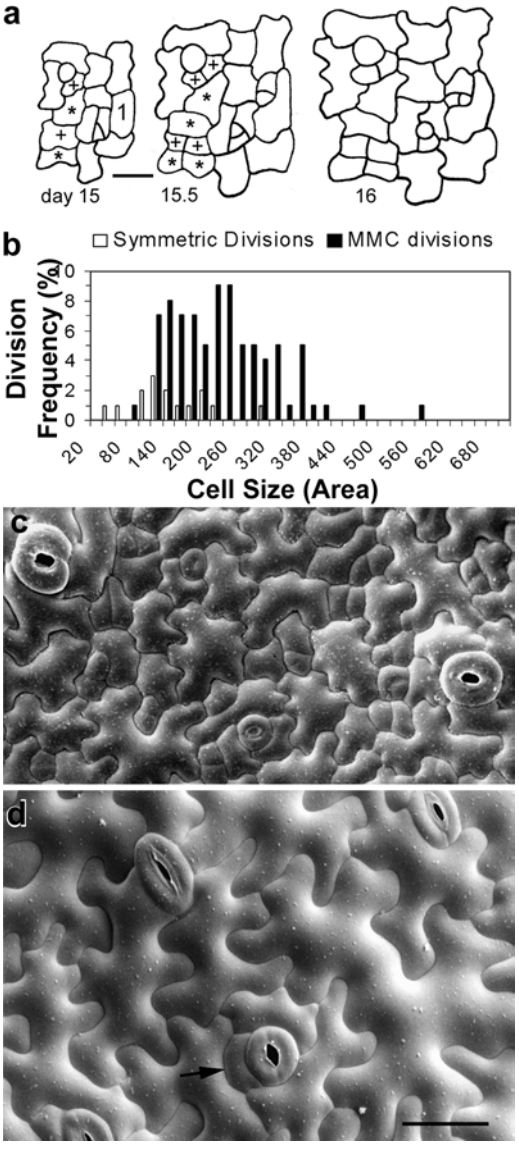

Fig. 3a–d Cell size and division. **a** Dental resin series from a developing first leaf of *Arabidopsis* showing symmetric and asymmetric divisions. Each of the four cells in the file at the left (+ and *) divided symmetrically by day 15.5. The top cell in the file was an NC. Cell "*1*" (day 15) was an MMC. Note the cell enlargement and increase in wall waviness by day 16. **b** Histogram showing the frequencies of divisions as a function of cell area (μm^2). Few divisions take place in cells larger than about 400 μm^2. **c, d** Cryo-scanning electron micrographs from a younger (**c**) and older (**d**) epidermis from the abaxial surface of cotyledons. As the epidermis ages, smaller, less sinuous cells decrease in number and become confined to stomatal complexes (*arrow*). Most other cells enlarge and become jigsaw puzzle-shaped pavement cells. Bars = 50 μm (**a**), 30 μm (in **d** for **c**, **d**)

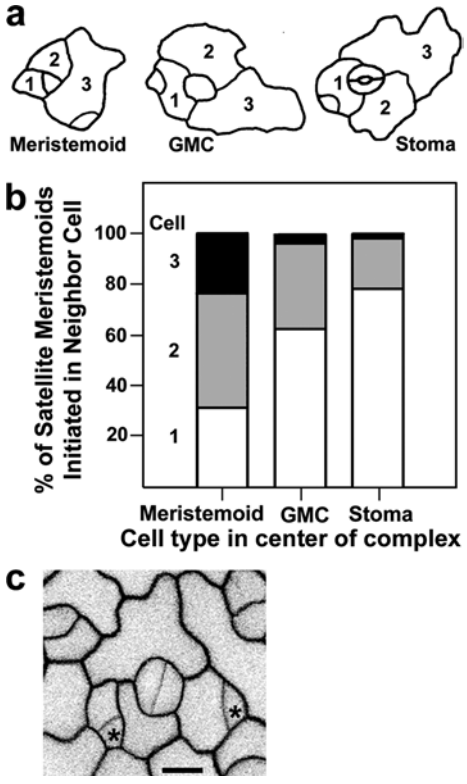

Fig. 4a–c Asymmetric divisions in NCs of *Arabidopsis*. **a** Diagram of different complexes at successively older stages of stomatal development. NC age is numbered (*1* youngest). Examples of satellite meristemoid formation are shown. **b** Relative division frequency decreases with NC age and with cell type in center of complex. *Black fill* in histogram bars represents oldest cells (cell 3), *white* youngest (cell 1). Data from 110 stomatal complexes whose NCs were followed using dental resin series. Only NCs that formed satellite meristemoids were included. **c** Apparent asymmetric divisions in two different NCs from one complex around a forming stoma. Confocal laser scanning microscope micrograph of plasma membrane GFP line. Fluorescence appears black due to inversion of image. Satellite meristemoids are indicated with an asterisk (*). The wall outlines suggest that the initial satellite meristemoid on the *left* divided asymmetrically. Bar = 5 μm

Similar NC division frequencies in young complexes

Next we evaluated the extent to which NC stage affected the probability of division. NC age (NC1 youngest) correlated with relative NC size. Complexes with a central meristemoid were typically younger than complexes containing a GMC or a stoma (Figs. 1b, 4a). In complexes surrounding a meristemoid, all three NCs were roughly equal in their propensity to divide asymmetrically (Fig. 4b). In older complexes, division competence became progressively restricted to smaller and younger NCs. Older NCs were more likely to differentiate into pavement cells, meaning that they did not divide, they enlarged, and their walls became sinuous (Fig. 3c–d).

Divisions per complex

We estimated how frequently asymmetric divisions occurred in the same developing complexes studied through time. Two-thirds of all WT complexes sampled had at least one NC that divided asymmetrically (Table 1). Twelve percent of complexes showed asymmetric divisions in two different NCs (Table 1; Fig. 4c). By pooling dental resin series from different stages of leaf

Table 1 Number of *Arabidopsis thaliana* NCs per individual complex that divided asymmetrically. Data are from a dental resin series of 100 complexes for each genotype. Percentages are means from complexes of different stages from 6- to 15-day-old cotyledons. The *tmm* complexes were all non-clustered

Total number of NCs that divided	Percent of all complexes	
	Wild type	*tmm*
0	42	6
1	46	69
2	12	21
3	0	3

Table 2 Frequency of asymmetric divisions in NCs of *Arabidopsis* as a function of relative NC size. Data derived from dental resin series of 196 WT complexes surrounding meristemoids, GMCs or stomata. From abaxial cotyledons

Relative NC size	Frequency of asymmetric division (%)
Smallest (NC1)	55
Medium (NC2)	15
Largest (NC3 & 4)	5
All NCs combined	24

development we estimated that about a quarter of all WT NCs divided asymmetrically (Table 2). Thus, many NCs did not form new stomata. More NCs divided asymmetrically in *tmm* than in WT complexes (Table 1).

Developmental progression in nuclear position

Neighbor cells were analyzed to determine whether nuclear position predicts an asymmetric division. A nucleus located away from the stoma might foreshadow the formation of a new meristemoid in a distal location. Nuclear position was scored as a function of NC stage as measured by relative NC size and by the type of cell in the center of the complex (Fig. 5). The demarcation of scoring zones was chosen because divisions in a proximal location might result in ectopic satellite meristemoids as may occur in *tmm* (Fig. 1c). However, proximal zones are typically smaller than distal ones (Fig. 5a). If nuclei were randomly distributed within the cell, then scoring might simply reflect the relative areas of the different zones. To test this we measured the area of each zone in a subset of NCs and then compared this predicted random distribution to that observed. The observed distributions were statistically different from random in the five stages that had at least half of all nuclei in a distal location (asterisks in Fig. 5b). Thus, there is a distal polarity to nuclear position that is present in the majority of NC stages.

Centrally located nuclei were characteristic of pavement cells. As larger NCs advanced in stage, nuclei moved from a random to a predominantly central location (compare three right bars with and without asterisks in Fig. 5b). These data support the existence of a developmental progression in nuclear position as a function of NC age from random (youngest NCs), to mostly distal (middle stages), to central.

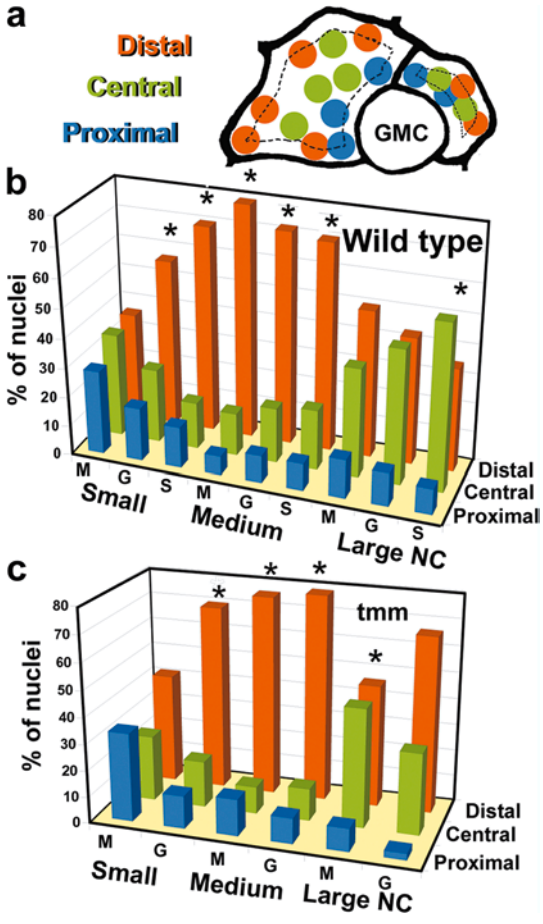

Fig. 5a–c Quantification of nuclear position in NCs. **a** Diagram showing how nuclear position was scored as proximal (*blue*), central (*green*) or distal (*orange*). Categories in small NCs (*right*) were assigned based on relative nuclear position. Scoring in larger NCs (*left*) was partly by the position of the edge of the nucleus. The *dotted line* is one mean nuclear radius from the cell walls. All non-peripheral nuclei were scored as central. **b** Histogram of WT. Most categories of NCs display a net distal nuclear polarity. NCs were categorized by whether they were adjacent to a meristemoid (*M*), GMC (*G*), or stoma (*S*) and by their relative size. The *asterisks* above the orange bars indicate that the observed distribution for the entire category differed statistically from a random distribution based on relative sector area (P≥95%). **c** As in **b**, but showing that *tmm* does not affect net nuclear polarity. The stomatal category was excluded because there were many clusters by the time stomata developed

Distal polarity and asymmetric division

Presumably, at least a fraction of NCs with a distal nucleus will divide asymmetrically. However, more NCs display a distal polarity than appear to divide asymmetrically. For example, 70–80% of medium-sized NCs had distal nuclei (Fig. 5b). In contrast, only about 15% of the medium-sized NCs sampled divided asymmetrically (Table 2). In addition, often all NCs in a complex

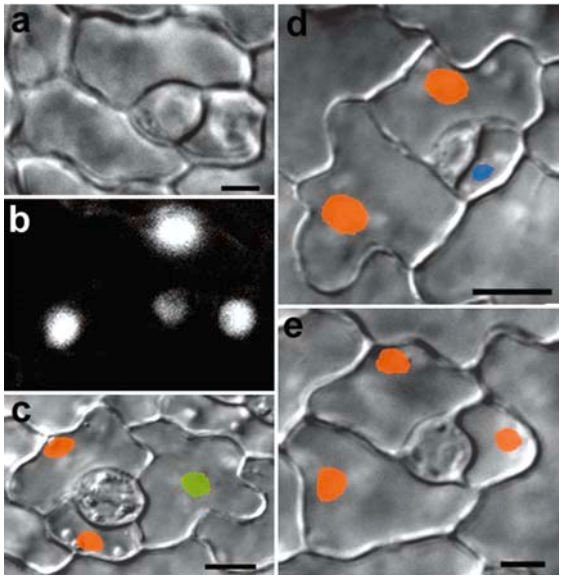

Fig. 6a–e Micrographs showing the mostly distal location of nuclei in NCs of *Arabidopsis*. All are WT except **e**, which is *tmm*. **a, b** Differential interference contrast (DIC) optics (**a**) and conjugate GFP nuclear fluorescence image (**b**) of a GMC complex. All three NCs have distally located nuclei. Images are representative of those used to collect data on nuclear position. **c–e** As in **a**, except that information about nuclear position from the corresponding GFP image is shown as a color-coded overlay on the DIC image. Color coding as in Fig. 5. The central cell is a developing stoma in **c**, a meristemoid in **d**, and a GMC in **e**. Bar = 5 μm (**a, e**), 10 μm (**c, d**)

had distal nuclei (Fig. 6a, b, e) even though 88% of all complexes formed no or just one satellite meristemoid (Table 1). Also, symmetric divisions often occurred in NCs (Fig. 3a), and newly formed daughter cells usually had distal nuclei (data not shown). This suggests that cells with distal nuclei can divide symmetrically as well as asymmetrically. These data support the idea that a distal nucleus does not necessarily predict an imminent asymmetric division.

Finally, it is not just the nucleus that is polarly localized. Chloroplasts and the bulk of the cytoplasm surround the nucleus, while the vacuole occupies the proximal part of the cell (Fig. 7). This cytological polarity in NCs resembles that in meristemoids that will divide asymmetrically (Fig. 2).

tmm does not disrupt nuclear polarity

Because the orientation of asymmetric divisions is randomized in *tmm* NCs, we investigated whether nuclear position was also disrupted. Although there were slight differences in nuclear position in *tmm* compared to the WT, the trends were similar in both genotypes. Most stages of small and medium *tmm* NCs displayed a statistically significant distal nuclear polarity (Figs. 5c, 6e).

In mature *tmm* leaves, about two-thirds ($63.8 \pm 8.3\%$ SD) of all stomatal units were clustered. To score nuclear

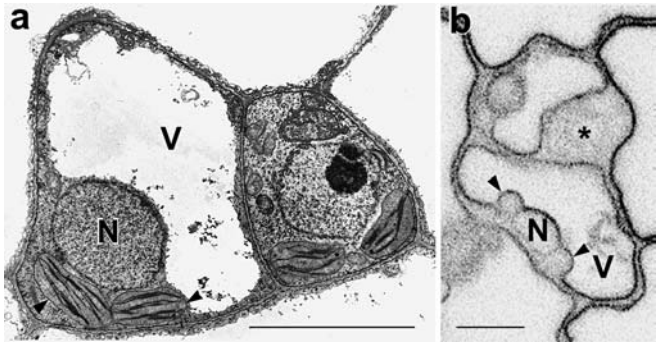

Fig. 7a, b Cytological polarity in NCs of *Arabidopsis*. Chloroplasts (*arrowheads*) are adjacent to the nucleus (*N*), and the vacuole (*V*) is close to the stomatal precursor. **a** Transmission electron micrograph of NC next to a GMC or late meristemoid. **b** Confocal micrograph of a GMC (*) complex using a tonoplast GFP marker. Both NCs shown display a distal cytological polarity. The fluorescent image was inverted so that the tonoplast appears as dark line. Chloroplasts are visible next to the nucleus at the lower left. Bars = 5 μm

position in developing leaves, the *tmm* sample included only young complexes that were isolated (all NCs were in contact with a central single meristemoid or GMC). This sampling was chosen because many non-isolated complexes had started to form clusters, which would complicate the evaluation of nuclear position. One possible reason why *tmm* nuclei were found to be normally placed is that isolated complexes are not as severely affected by the *tmm* defect as those already clustered (variable expressivity). To address this possibility, we scored the placement of *tmm* satellite meristemoids in developing leaves that were the same age as those used for the analysis of nuclear position. Of all first satellite meristemoids ($n = 403$), 30.3% formed in contact with the pre-existing stoma/precursor, and the remainder were placed away (Fig. 8). These data demonstrate that the NCs examined in this study are affected by *tmm* and are consistent with the hypothesis that *tmm* randomized the plane of division.

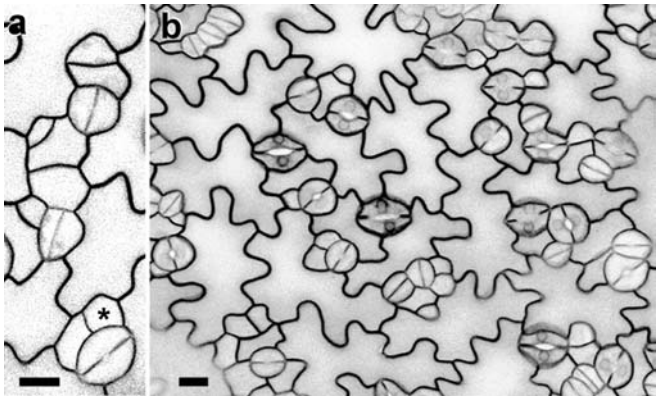

Fig. 8a, b Abaxial epidermis of developing *tmm* leaf. Image techniques as in Fig. 4c. **a** Incorrect asymmetric division planes in NCs (* shows ectopic first satellite meristemoid). **b** Most complexes are clustered or becoming so. In mature leaves, about two-thirds are clustered. Bars = 10 μm

Together these results suggest that the *tmm* mutation does not disrupt the net distal nuclear polarity in NCs.

Discussion

Arabidopsis stomatal patterning requires the orientation of asymmetric divisions in cells that neighbor a pre-existing stoma or precursor. This process is disrupted by the *too many mouths* mutation (Geisler et al. 2000). Our results suggest that a polarly located nucleus does not necessarily predict an asymmetric division in NCs and that *TMM* is not required for normal nuclear migration.

Small tiles in the mosaic and stomatal number

Because the asymmetric divisions of NCs give rise to many *Arabidopsis* stomata (Geisler et al. 2000), we studied NCs of different ages and sizes to determine those likeliest to divide. All NCs next to a meristemoid produced stomata with roughly equivalent frequencies. Only later in development did asymmetric divisions become confined to the youngest NC (Berger and Altmann 2000). There thus appears to be a dynamic equilibrium in division frequency that shifts with NC stage and age.

Stomatal formation takes place throughout leaf expansion and is asynchronous and iterative (Geisler and Sack 2002). Stomatal production depends upon the presence of division-competent cells. Almost all of the epidermal cells sampled that divided during leaf growth were smaller than about 400 μm^2 in area. A comparable threshold was obtained using cell cycle reporters in fixed leaves (Donnelly et al. 1999). Cells of this size include NCs as well as isolated cells, and both are distributed throughout much of the young epidermis. As the leaf undergoes mosaic growth, the smaller cells become progressively restricted to stomatal complexes. These small cells, which can be considered small tiles in a mosaic, provide a reservoir of cells capable of dividing asymmetrically throughout leaf growth. Thus, the spatial distribution of small tiles in the mosaic changes during leaf growth in a pattern that coincides with where stomata are likely to form.

Conversely, the larger size of most epidermal cells correlates with a lack of division and with differentiation into pavement cells. Melaragno et al. (1993) found that cells in a mature *Arabidopsis* epidermis that had 2C levels of DNA had a mean area of 480 μm^2. Larger cells were endopolyploid, with DNA levels proportional to cell size. Figures in that paper show that the small, 2C cells were the smallest NCs. Although small 2C cells have been referred to as pavement cells (Melaragno et al. 1993; Traas et al. 1998), it remains to be determined whether they express fate markers of mature pavement cells. In any case, the reduced number of asymmetric divisions in large NCs may correlate with endoreduplication and their differentiation into pavement cells. Stomatal number is partly regulated by the size of the pool of cells that can proliferate. This pool appears to be filled by symmetric and asymmetric divisions, and progressively drained by differentiation.

This model is also consistent with the proposed function of TMM in regulating whether or not stem cells divide (Nadeau and Sack 2002b). The pattern of *TMM* expression is at least partly correlated with division competence. *TMM* is expressed in smaller NCs and in putative isolated MMCs but not in pavement cells. Loss of *TMM* function results in ectopic divisions in NCs but does not appear to affect pavement cells (Geisler et al. 2000).

Distal nuclear polarity and asymmetric division

In many plant cells, including meristemoids, the nucleus migrates to a position that predicts the site of a future asymmetric division (Galatis and Mitrakos 1979; Kennard and Cleary 1997; Zhao and Sack 1999; Smith 2001). The direction of nuclear migration is often determined by spatial cues generated by intercellular signaling (Fowler and Quatrano 1997). A distal nucleus in an *Arabidopsis* NC is an appropriate location for forming a new, correctly patterned meristemoid. However, our data suggest that more NCs have distal nuclei than are likely to divide, and that a distal position does not necessarily predict the occurrence of an asymmetric division.

Why would NCs display a distal nuclear polarity if only a fraction is likely to divide asymmetrically? One possibility is that the net distal polarity results from the addition of two populations, those nuclei that mark an imminent asymmetric division, and those located distally by chance. This combination would produce a distal majority because of the greater area of the distal scoring zone. An alternative is that the movement of nuclei to a distal position is actively regulated as a function of stage. For example, NCs might receive and interpret spatial cues that are used as landmarks for nuclear migration but not necessarily for establishing the site of division.

We hypothesize that a distal nucleus is a marker for NC division competence and limited plasticity in cell fate. The categories of NCs with net distal nuclei (Fig. 5) are also those most likely to divide asymmetrically. Small NCs can also divide symmetrically or differentiate into pavement cells. By contrast, those NCs that are least likely to divide, large NCs next to stomata, have centrally located nuclei. Central nuclei are also found in large, endoreduplicated pavement cells that usually do not divide and that are already differentiated. There thus appears to be a developmental progression from small division-competent NCs to those differentiating as pavement cells.

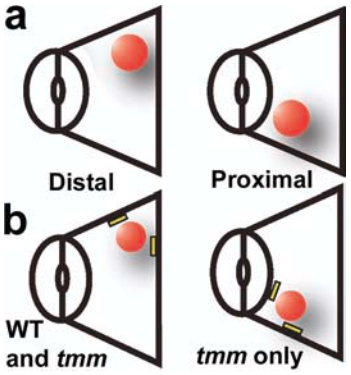

Fig. 9a, b **a** Summary for nuclear position. Nuclei are mostly distal in younger WT and *tmm* NCs but can also be proximal or central (latter not shown). **b** Illustration of hypothesis that *tmm* allows the division site to incorporate a proximal nucleus

The range of events found in *Arabidopsis* NCs differs from those in developing monocot stomatal complexes where both nuclear migration and asymmetric division invariably take place (Kennard and Cleary 1997; Smith 2001). In contrast, many *Arabidopsis* NCs never divide asymmetrically and the fraction that does so is under genetic, environmental, developmental and positional control (von Groll and Altmann 2001; Nadeau and Sack 2002a). These differences might explain the lack of one-to-one correspondence between a distal nucleus and an asymmetric division in NCs.

TMM and NC division

Geisler et al. (2000) showed that *tmm* randomizes the plane of asymmetric divisions in NCs, resulting in ectopic satellite meristemoids and stomata in contact. TMM may function in a pathway that uses intercellular signaling to place new divisions in a distal location in NCs (Nadeau and Sack 2002b). One process that might be controlled by TMM is the position and movement of nuclei to a distal location, a process that could be disrupted by *tmm*. However, *tmm* NCs displayed essentially the same nuclear polarity profile as the WT.

These results suggest several possible mechanisms by which positional signals could regulate division orientation in NCs. One scenario is that cells with proximal nuclei are normally prohibited from dividing, but do divide in *tmm* because negative signals cannot be perceived (Fig. 9). Another possibility is that nuclear location is not critical for division orientation in either *tmm* or in the WT. The primary role of cell signaling might be to orient the plane of the new cell wall rather than to position the nucleus. The plane of division might be correctly placed in the WT even around nuclei close to a guard cell or precursor, but be randomly placed and thus ectopic in *tmm*.

In addition to orientation, TMM also regulates division frequency because more NCs per complex divide asymmetrically in *tmm* than in the WT. So TMM may limit the size of the pool of NCs that can function as stem cells or it may restrict a commitment to asymmetric division. This limiting function of TMM also seems to be independent of the distal nuclear polarity.

In summary, we have shown that more NCs have nuclei located away from a stoma or precursor than are estimated to divide asymmetrically. The presence of a distal nucleus may not always predict an asymmetric division, but occurs in NC classes most likely to divide. Divisions are dispersed in NCs around a meristemoid but later become restricted to the smallest NC. The position of the NC nucleus may be actively regulated, perhaps by intercellular signaling. *TMM* may be unnecessary for nuclear positioning and may primarily regulate the placement of the division site itself.

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