Clonal Analysis of Stomatal Development and Patterning in Arabidopsis Leaves

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Cell lineage has been used to explain the stomatal distribution in several plant species. We have used transgenic plants carrying a 35S::GUS construct that produces clonal sectors to analyze the possible role of cell lineage during the establishment of stomatal patterning in Arabidopsis leaves. The analysis of sectors ranging from two to eighteen cells supports the conclusion that most stomatal complexes derive from a single and immediate precursor cell through a stereotyped pattern of three unequal cell divisions followed by a final equal one. In addition, it shows that the successive cell divisions take place at a constant angle (approximately 60°) with respect to the previous one. Interestingly, this angular dimension shifts from 60° to 0° in the last cell division that gives rise to the stoma. These sectors also reveal the development of both clockwise and counterclockwise patterns of cell divisions during stomatal development in approximately equal numbers. Our clonal analysis indicates that cell divisions involved in the development of stomatal complexes are probably the last ones contributing to epidermal growth and development. Finally, the stereotyped pattern of cell divisions that culminates in the formation of stomatal complexes indicates that cell lineage plays a very important role during stomatal pattern establishment.

Key Words: Activator element; anisocytic stomatal complex; Arabidopsis; clockwise; clonal analysis; counterclockwise; epidermis; monoclonal; polyclonal; stomatal pattern.

INTRODUCTION

The stoma is a two-celled epidermal structure that regulates gas exchange between the plant and the atmosphere (Taiz and Zeiger, 1991). It consists of a pair of guard cells (GCs) that delimit a stomatal pore, and it is surrounded by a variable number of subsidiary cells (SCs) with which GCs exchange water and ions to open or close the pore (Taiz and Zeiger, 1991). As a general rule, stomata never develop adjacent to one another, but instead they are separated by a number of intervening epidermal cells.

Two main mechanisms have been proposed to explain the nonrandom stomatal pattern in the leaves of dicotyledonous plants. One mechanism proposes that stomata or their precursors (meristemoids, Ms) release inhibitory factors that prevent the same cell fate in the immediate vicinity (Bünning and Sagromsky, 1948; Korn, 1972, 1981; Marx and Sachs, 1977; Kagan et al., 1992). The two mechanisms are not mutually exclusive, that is, both inhibitory factors and cell lineage could account for the stomatal pattern in a number of plant species (Sachs, 1994).

In Arabidopsis, many stomata are surrounded by three SCs (see, e.g., Berger and Altmann, 2000; Serna and Fenoll, 2000a). This five-celled structure is called an anisocytic stomatal complex (Metcalfe and Chalk, 1950). Non-anisocytic complexes, such as those consisting of one stoma surrounded by more than three SCs have also been described in Arabidopsis (Yang and Sack, 1995; Serna and Fenoll, 1997; Zhao and Sack, 1999; Geisler et al., 2000; Serna and Fenoll, 2000a). In the adaxial epidermis of Arabidopsis leaves (Landsberg erecta ecotype), most stomatal complexes are adjacent to one another, that is, most stomata of neighbour complexes are separated by two cells (Serna and Fenoll, 2000a). Knowing the cell division patterns that culminate in the formation of these stomatal complexes is necessary to understand the possible role of...
cell lineage and/or cell interactions during the establish-
mament of stomatal pattern.

By using nondestructive techniques to monitor cell divi-
sion patterns, the development of stomatal complexes in
two different Arabidopsis ecotypes has recently been de-
scribed. In the primary leaves of C24 (abaxial side), the
stomatal precursors execute a fixed pattern of three unequal
cell division followed by a final equal one, thus generating
the stoma and the three SCs (Berger and Altmann, 2000;
reviewed by Brownlee, 2000 and Serna and Fenoll, 2000b;
Fig. 1). In contrast, stereotyped cell lineages do not seem to
be involved in the production of stomata in Columbia (in
g1 genetic background) leaves and cotyledons (Geisler et al.,
2000). Therefore, stomatal lineages vary depending on the
ecotype under study.

To know the contribution of cell lineage during the
establishment of stomatal patterning in the adaxial epider-
mis of Landsberg erecta leaves, we have analyzed clones of
epidermal cells. Clones were revealed by Activator (Ac)
excision from a 35SGUS::Ac construct, which allows GUS
expression. Our clonal analysis allows describing the num-
er and orientation of the cell divisions that occur during
stomatal complex development, establishing a very impor-
tant role for lineage in stomatal patterning. The present
work provides a structural framework for the analysis and
interpretation of previously isolated stomatal mutants (Wei
et al., 1994; Yang and Sack, 1995; Schneider et al., 1997;
Berger and Altmann, 2000; Gray et al., 2000).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Sector analysis was carried out in the Arabidopsis thaliana
Landsberg erecta ecotype transformed with a fusion of the 35S
promoter to the β-glucuronidase gene (uidA), interrupted by the
maize Ac transposon (Lawson et al., 1994).

Seeds were vernalized at 4°C for several days. They were germi-
nated on soil at a photon flux density of 70 μmol m⁻² s⁻¹, at
20–25°C and a 16-h photoperiod. Seedlings were maintained under
these growth conditions and covered with plastic wrap for one
week. Then, the plastic was removed and the plants were grown
under the same conditions for three more weeks. Four week-old
plants had mature leaves 1 and 2 and were used for the clonal
analysis.

Histochemical GUS Assays

Histochemical assays of GUS activity were conducted as previ-
ously described (Jefferson et al., 1987; Schrammeijer et al., 1990),
with some modifications. Plants were incubated at 37°C overnight
in 50 mM NaH₂PO₄, pH 7.0, containing 1 mM 5-bromo-4-chloro-
3-indoyl-β-D-glucuronide (X-gluc), 0.5 mM K₃[Fe(CN)₆], 0.5 mM
K₄[Fe(CN)₆], 0.1% (w/v) Triton X-100, 10 mM Na₂-EDTA. After
histochemical GUS assays, leaf samples were cleared by boiling for
5 min in 95% ethanol and fixed overnight in lactophenol. Lacto-
phenol was removed and leaves were stored in 95% ethanol at 4°C.

Sector Analysis

Random excision of the Ac element from the 35SGUS::Ac
construct (Lawson et al., 1994) in a single cell leads to GUS
expression in all descendants of this precursor cell if the 35S
promoter is active in all cells derived from the cell in which the
excision event took place. Assuming that sectors derive from a
single Ac excision, epidermal blue-stained sectors can be used to
dissect cell lineage in the leaf epidermis.

For the purpose of this work, it is a requirement that the Ac
excision event takes place during stomatal complex development.
For this reason, a transformed line that displayed small sectors
(from one to eighteen cells) was selected. A total of 122 mature
leaves 1 and 2 were stained for GUS activity. Typically, between
five and nine sectors were found and analyzed in every sampled leaf
(adaxial epidermis). Occasionally, leaves showing only one/two or
more than twenty sectors were also found and their sectors were
also analyzed. Sectors (n = 899) were classified into five classes
depending on their cell number: two-celled, three-celled, four-
celled, five-celled and more than five-celled. Every sector was
described in detail according to the cell types present (GCs, SCs
and/or pavement cells) and to the number of stomata included.

Angle Measurements

Angles defined by intersecting SC walls were measured on
typical four-celled sectors (SC2, SC3 plus stoma). Angles from a
total of 45 four-celled sectors were measured. The angle formed
between a M division plane with respect to the previous cell
division plane was inferred from the angle formed between the cell
walls produced by such divisions (see Fig. 1). Thus, the angle formed
between SC1/SC2 intersecting walls represents the orienta-
tion between the M1 division plane (that formed SC2 and M2)
and the previous division plane that produced SC1 and M1.
Similarly, the angle between SC2/SC3 intersecting walls represents
the relative orientation between the division plane of M2 (forming
SC3 and M3) and the previous M1 division plane (that formed SC2
and M2).

In each four-celled sector, SC3 was identified because of the
constant orientation of the guard mother cell (GMC) division plane
with respect to the M2 division plane (measured as described
below). SC1 in a four-celled sector is defined as the unstained SC
contacting the stoma, assuming that such cell is clonally related
with this sector. SC2 is the remaining SC in the sector.

The angle formed between the GMC and the M2 division planes
were inferred from the angle formed between the cell wall common
to the two GCs and the SC3 wall that contacts the stoma, in
three-celled sectors. Angles from a total of 101 three-celled sectors
were measured.

All measurements were made on Nomarski optics micrographs,
at a 1,000× magnification. We assume that the angles between cell
walls established by a given division are not significantly modified
during stomatal complexes development.

Nomarski Microscopy

Leaves were mounted on microscope slides under coverslips.
Sectors were examined and photographed using Nomarski optics in
a Leica DM1000 inverted microscope with a 40X or 60X objective.
RESULTS

Ac Excision in the 35SGUS::Ac Line

Ac excision from the 35SGUS::Ac construct leads to GUS expression in the cell where the transposition event takes place and in all its descendants. Thus, assuming that sectors derive from a single Ac excision, the sector size indicates the moment in which the Ac excision took place. Larger blue-stained sectors indicate earlier Ac excisions, while smaller sectors reveal later excisions.

As shown in Table 1 and Table 2, of a total of 899 sectors analyzed, 676 (75.2%) were two-celled, 101 (11.2%) were three-celled, 45 (5.0%) were four-celled, 31 (3.5%) were five-celled and 46 (5.1%) consisted of more than five cells. Thus, larger sectors were found at lower percentages, revealing that in this line Ac excision takes place preferentially at very late stages during leaf epidermal development. No sectors consisting of trichomes plus some of their accessory cells were found, as expected from the fact that trichome differentiation is an early event during leaf growth and development (Larkin et al., 1996). No sectors composed of epidermal cells located above the main vein were found.

Analysis of Two-Celled Sectors

A common feature that takes place during stomatal development in all the plant species studied is that the GMC divides symmetrically, giving rise to two cells that stop dividing and differentiate into the pair of GCs (stoma). This feature predicts that at least some of the two-celled sectors identified should be formed by paired GCs. Of a total of 676 two-celled sectors analyzed, 672 (99.4%) were stomata (Table 1, Fig. 2A). Two sectors (<1%) encompassed a pair of SCs that belonged to a nonanisocytic complex consisting of a stoma surrounded by four SCs (Fig. 3A, left; see Discussion for interpretation). The two remaining sectors (<1%) consisted of two SCs of adjacent anisocytic complexes (Fig. 3B, left; see Discussion).

These sectors reveal that in most of the cases, Ac excision took place in the immediate stomatal precursor cell (GMC or M3) which, after dividing, gave rise to two blue-stained GCs (see Fig. 1). Therefore, the analysis of two-celled sectors confirms the largely known: the stoma derives from a single cell.
**Analysis of Three-Celled Sectors**

If the stoma and some of its three surrounding SCs derive from a single and immediate precursor cell, then it is expected that three-celled sectors should include one stoma and one SC (SC3). All 101 three-celled sectors analyzed had this configuration (Table 1, Fig. 2B). Considering that the stoma derives from the GMC (see previous section), we interpret these sectors as the result of excisions from the immediate precursor cell (M2) of both the GMC and the SC3 (see Fig. 1). These sectors also indicate that the stained SC3 did not undergo further cell divisions.

The analysis of three-celled sectors indicates that the GMC and the SC3 are sister cells. This finding allowed us to define the orientation of the GMC division plane relative to the previous one (Fig. 3C). Of a total of 101 GMC divisions planes scored, 77 (76.2%) were approximately parallel, with a value of 0.0° ± 4.2° (mean ± SD), to the preceding one. The remaining 24 (23.8%) GMC division planes did not show any preferential orientation. This relationship between cell division planes allowed us to define the SC3 as that in which the cell wall that makes contact with the stoma is parallel to the GMC division plane.

**Analysis of Four-Celled Sectors**

Sectors composed of four epidermal cells were also found. All 45 four-celled sectors scored consisted of one stoma and two SCs of the same anisocytic stomatal complex (Table 1, Fig. 2C). Since the three-celled sectors (stoma plus SC3) derive from the M2 (see previous section), we interpret that these four-celled sectors arise from Ac excisions in the mother cell of both the M2 and the SC2, that is, in the M1 (see Fig. 1). This interpretation implies that the unstained SC (SC1) that makes contact with the stoma in the four-celled sector is clonally related to this sector (see Fig. 1). In addition, these sectors show that both the SC2 and SC3 did not undergo further cell divisions.

The relationship between the plane of the cell division that generates the stoma and the plane of the preceding division allowed us to identify the youngest SC (SC3) of the anisocytic stomatal complex (see previous section and Fig. 3C). This finding made it possible to experimentally address, by analyzing four-celled sectors, the direction of the serial divisions that culminate in the formation of the anisocytic complex. If the unstained SC (SC1) is on the left of the youngest SC (SC3) the twist is clockwise (Fig. 3D, left). Conversely, a counterclockwise twist is revealed by...
the presence of the unstained SC on the right of the youngest SC (Fig. 3D, right). Of a total of 45 four-celled sectors analyzed, 21 (47%) followed a clockwise sequence and 24 (53%) followed a counterclockwise twist.

Four-celled sectors also allowed us to analyze the orientation of the second and third cell division planes during the development of the anisocytic stomatal complex relative to the preceding one (Fig. 1) (see Material and Methods). All second \((n = 45)\) and third \((n = 45)\) cell division planes analyzed were oriented at, respectively, \(60.0^\circ \pm 6.0^\circ\) and \(60.0^\circ \pm 5.9^\circ\) (mean \(\pm SD\)), relative to the planes of the former division.

### Analysis of Five-Celled Sectors

Five-celled sectors were also identified. Of 31 of these, 27 (87%) were composed of one stoma and its three SCs (SC1, SC2, SC3), that is, they consisted of the entire anisocytic stomatal complex (Table 1, Fig. 2D). Four sectors (13%) stained partially two anisocytic complexes: they consisted of one SC from one of the complexes and the stoma and two SCs from the other one (Fig. 3B, right; see Discussion for interpretation).

The fully stained anisocytic complexes derive from a single cell (Fig. 2D). In addition, since four-celled sectors (stoma plus two SCs) derive from the M1 (see previous section), we interpret that five-celled sectors arise from \(Ac\) excisions in the mother cell of both the M1 and the SC1 (see Fig. 1). These sectors consisting of the entire anisocytic complex also indicate that none of its SCs (SC1, SC2, and SC3) underwent further cell divisions. Thus, they represent the last stomatal complex formed within a given lineage (see next section).

### Analysis of More Than Five-Celled Sectors

Sectors composed of more than five cells were also found (Fig. 3A, right and 4). These sectors were classified according to the number of stomata that they contained (Table 2).

### TABLE 2
Characterization of More Than Five-Celled Sectors Induced by \(Ac\) Excision in the Leaf Epidermis of \(Arabidopsis\)

<table>
<thead>
<tr>
<th>Number of stained stomata</th>
<th>Cell number (range)</th>
<th>All SCs stained</th>
<th>Some SCs unstained</th>
<th>Total sectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>6</td>
<td>11</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Two</td>
<td>7-11</td>
<td>12</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>Three</td>
<td>10-15</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Four</td>
<td>18</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

SCs = subsidiary cells.
Of a total of 46 sectors analyzed, 11 (24%) exhibited only one stained stoma. Two sectors consisted of one stoma surrounded by four SCs (Fig. 3A, right), indicating that these nonanisocytic complexes derive from a single cell. The nine remaining sectors were composed of one fully stained anisocytic complex plus one SC of an adjacent complex (n = 6) (Fig. 4A) or one pavement cell (n = 3) (Fig. 4B). Since most anisocytic complexes derive from a single cell (see previous section), we interpret these sectors composed of one anisocytic complex and one SC of an adjacent complex to be derived from Ac excisions in this SC, with the fully stained anisocytic complex derived from the stained SC. Thus, these fully stained complexes are, at least, secondary. Two interpretations may explain those sectors consisting of one anisocytic complex and one pavement cell. They may derive from Ac excisions in a protodermal cell that divided asymmetrically giving rise to two cells with different fates: one precursor cell of one anisocytic complex and one pavement cell. However, it is also formally possible that these sectors derive from Ac excisions in a protodermal cell that gave rise to one anisocytic complex. A cell division from a SC of this stomatal complex so that the cell division plane did not intersect the stoma would produce the pavement cell. This second interpretation predicts the formation of specific sectors, such as two-celled sectors consisting of one SC plus one pavement cell. Such sectors were never found.

The remaining sectors (76%) included more than one stoma. Thirty-two sectors (70%) showed two stained stomata. Seven of these sectors consisted of two fully stained anisocytic stomatal complexes (Fig. 4C). Thirteen sectors were composed of two anisocytic complexes, one of them exhibiting one (n = 6) (Fig. 4D) or two (n = 7) (Fig. 4E) unstained SC/SCs. One sector showed two fully stained anisocytic stomatal complexes plus one pavement cell (Fig. 4F). The eleven remaining sectors consisted of two stomata sharing one SC. One of the stomata was, in all cases, surrounded by three stained SCs, and the other one was either surrounded by three stained SCs (n = 4) (Fig. 4G) or had one (n = 3) (Fig. 4H) or two (n = 4) (Fig. 4I) unstained SC/SCs. Two sectors (4%) exhibited three stained stomata. In one of the sectors every stoma shared one SC with the nearest stoma. One of the stomata placed in one of the extremes was surrounded by one stained and two unstained SCs, and the other two stomata were flanked by three stained SCs (Fig. 4J). The other sector consisted of three fully stained anisocytic complexes (Fig. 4K). Finally, one sector (2%) showed four stained stomata. This sector consisted of a linear group of four anisocytic stomatal complexes, in which one of the complexes located at a sector terminus exhibited two unstained SCs (Fig. 4L). All sectors containing two or more stomata, one of which was surrounded by unstained SCs, are interpreted as the result of Ac excisions during the development of the partially stained structure, with the fully stained complexes sequentially derived from the partially stained ones (see Fig. 1). Thus, complexes of at least quaternary order are formed.

The sectors consisting of two, or more than two fully stained anisocytic complexes, may derive from Ac excisions in a protodermal cell that divided before entering the stomatal pathway. This cluster of protodermal cells would produce several primary stomatal complexes in direct contact. Alternatively, these sectors may derive from Ac excisions in a protodermal cell (or SCs) that produced higher order stomatal complexes (secondary and higher order).

**DISCUSSION**

**The Last Stage during Epidermal Growth and Development: The Formation of Stomatal Complexes**

The sectors analyzed differed in cell composition and size. However, most sectors (99.6%) shared a common characteristic: the presence of stomata. This observation strongly suggests that stomatal formation, which starts near the leaf tip and proceeds basipetally (Larkin et al., 1996), is not only the last event that takes place during stomatal complex development, but it is also the last event occurring during epidermal growth and development. Therefore, at the late stage of leaf development when the Ac excisions are occurring, essentially, every dividing cell is destined to produce stomatal lineages. This view is also supported by analysis of cycling cells, which gradually become restricted to Ms (Donnelly et al., 1999). Donnelly et al. (1999) identified a developmental stage in which the majority of cycling cells are Ms distributed throughout the whole leaf blade. Considering that the sectors we identified are scattered through out the leaf blade, and that most of them include stomata, they must have arisen by Ac excisions during this late developmental stage.

Is it possible to define a stereotyped cell division pattern during this late stage of epidermal growth and development? Our clonal analysis performed in the adaxial epidermis of Landsberg erecta leaves confirms the two main features recently disclosed by monitoring cell division patterns in the abaxial epidermis of C24 leaves (Berger and Altmann, 2000). They are: (1) the development of the vast majority of the stomatal complexes through a stereotyped pattern of three unequal cell divisions followed by a final equal one, which culminates in the formation of complexes derived from a single and immediate precursor cell (monoclonal complexes); and (2) the formation of higher order stomatal complexes. We have found evidence of quaternary (or higher order) complexes, since one identified sector consisted of a linear group of four anisocytic complexes, one of which, placed in one of the extremes, was partially stained (Fig. 4L). Another sector encompassing four fully stained anisocytic complexes was previously described (Serna and Fenoll, 2000a) and it may also support the formation of these quaternary complexes in the adaxial epidermis of Landsberg erecta. The stereotyped cell division pattern that characterizes stomatal development in Landsberg erecta and C24 contrasts with those described in Columbia (in gl1 genetic background); in this ecotype the
number of unequal divisions that take place before stomata formation varies from zero to three (Geisler et al., 2000).

A very low number of sectors that unequivocally support the development of anisocytic stomatal complexes derived from at least two different lineages (polyclonal stomatal complexes) was also found (0.7%, $n = 899$). They were the two-celled sectors staining two SCs of adjacent stomatal complexes, and the five-celled sectors staining partially two neighbor stomatal complexes (Fig. 3B). They indicate that at least one of the partially stained complexes is polyclonal.

**FIG. 4.** Drawings of Ac-induced sectors that stained more than five cells. (A-B) Sectors staining only one stoma. (C-I) Sectors exhibiting two stained stomata. (J-K) Sectors showing three stained stomata. (L) Sector staining four stomata. (A, D, E, H, I, J, L) Sectors composed of two or more complexes, one of which was partially stained, are interpreted as derived from Ac excisions during the development of the partially stained complex, with the fully stained complexes sequentially derived from the partially stained ones. These sectors reveal the formation of at least (A, D, E, H, I) secondary, (J) tertiary and (L) quaternary complexes. Several interpretations account for the development of sectors consisting of (C, F) two or (K) more fully stained complexes: they might derive from a monoclonal group of protodermal cells that enter the stomatal pathway producing clusters of primary complexes. Alternatively, they might derive from a stomatal precursor cell that produces higher order of complexes. Grey-color indicates GUS activity.
Do these sectors mean that 99.3% of the anisocytic complexes in the adaxial epidermis are monoclonal? In a previous clonal analysis of anisocytic stomatal complexes placed on the borderline of Aci-induced sectors spanning the length of the adaxial epidermis, 10.7% (n = 75) of the complexes were bisected by sector boundaries, indicating that they are monoclonal (Serna and Fenoll, 2000a). Since both monoclonal and polyclonal models can explain the remaining 89.3% fully stained anisocytic complexes, it was concluded that at least 10.7% of the anisocytic stomatal complexes in Landsberg erecta are monoclonal (Serna and Fenoll, 2000a). Interestingly, a similar percentage (13%) of the five-celled sectors analyzed in this work revealed development of polyclonal complexes. By integrating such clonal analyses we conclude that approximately 87% of the anisocytic complexes placed in the adaxial epidermis of Landsberg erecta leaves derive from a single precursor cell through the recently established cell division pattern (Fig. 1). This implies that some of the partially stained complexes cannot be interpreted as the result of late excisions during the development of monoclonal complexes, but, instead, they must be explained by models that culminate in the formation of polyclonal complexes. Cell division patterns characterized by stomata formation after the second or first unequal cell division, and thus preventing the formation of a full and clonal complement of SCs, have been described (Zhao and Sack, 1999; Berger and Altmann, 2000; Geisler et al., 2000) and are a reasonable explanation for the development of these partially stained complexes. This stomata formation after the second or first unequal cell division might also explain the development of polyclonal complexes detected by analyzing borderlines of clonal sectors that spanned the length of Landsberg erecta leaves (adaxial epidermis) (Larkin et al., 1996; Serna and Fenoll, 2000a).

Particularly interesting is the finding of sectors consisting of two or more stomata separated by two SCs, one of which (always placed in one of the extremes) was surrounded by unstained SC/SCs (Fig. 4D, E, and L), and of sectors composed of one fully stained anisocytic complex plus one SC of an adjacent complex (Fig. 4A). These sectors suggest that some SCs divide in such a way that the cell division plane does not contact the stoma. The daughter cell placed away from the stoma assumes then stomatal complex precursor cell identity, giving rise to a new anisocytic complex in direct contact with the previously formed one and preserving a distance of two cells between stomata. This situation differs from that supported by sectors consisting of two or more stomata sharing SCs, in which one of the stomata was surrounded by unstained SC/SCs and placed in one of the extremes of the sector (Fig. 4H, I, and J). These sectors suggest that some SCs acquire stomatal complex precursor cell identity before dividing, leading to the formation of structures in which stomata are separated by one SCs instead of two. This cell division pattern has been previously described in the abaxial epidermis of C24 (Berger and Altmann, 2000).

Orientation of the Successive Cell Division Planes during the Development of the Anisocytic Stomatal Complex

A simple visual examination of Arabidopsis leaves (see, e.g., Fig. 4 in Serna and Fenoll, 2000a) reveals a large variability in the orientation of the stomatal complexes. This suggests that the first cell division in the stomatal pathway is randomly oriented. However, successive cell divisions from the M1 take place at a very constant angle with respect to the previous division planes (Fig. 1). The M1 divides so that its division plane is oriented approximately at a 60.0° ± 6.0° angle with respect to the preceding one and intersects it. Similarly, the cell division plane formed from the M2 is oriented approximately at 60.0° ± 5.9° with respect to the previous one, and intersects the two former cell divisions planes. This strict orientation of the cell division planes gives rise to a M (M3), and thus to a future stoma, that makes direct contact with the three immediately clonally related cells: SC3, SC2, and SC1. In contrast, the GMC division plane is oriented, in 76.2% of the cases, at approximately 0.0° ± 4.2° with respect to the preceding one.

This suggests some kind of mechanism that regulates the conserved angle observed in the second and third cell divisions that take place during stomatal development, and that establishes a new angle for the last cell division that culminates in the formation of the stoma. The nature of this mechanism is unknown.

Two Directions for the Cell Division Pattern That Gives Rise to the Anisocytic Complex: Clockwise and Counterclockwise

Our clonal analysis indicates that the stereotyped cell division pattern represented in Fig. 1 takes place in two directions: clockwise and counterclockwise (Fig. 3D). The two twists occur at very similar percentages. A similar situation has been reported for the development of stomatal complexes in Raphanus (Pant and Kidwai, 1967) and roots in Azolla (Gunning et al., 1978).

To determine the direction of the twist, we defined the youngest SC (SC3) as that in which the cell wall making contact with the stoma was parallel to the GMC division plane (Fig. 3C). It should be noted that this is true in just 76.2% of the cases. Therefore, the percentages of both clockwise and counterclockwise sequences should be interpreted with caution, since errors in the determination of the youngest SC (SC3) could affect such percentages. In any case, our data demonstrate that the pattern of cell divisions is not unidirectional, but that both clockwise and counterclockwise patterns take place during stomatal development in Arabidopsis leaves.

It has been shown that the developmental program undergone by the precursor cells (protodermal cells) of the primary complexes serves as a template for some of their SCs (Berger and Altmann, 2000). Is the direction of the twist...
also included in this basic program and, therefore, conserved within a particular group of complexes produced by the same lineage? This question remains to be answered.

**Monoclonal and Polyclonal Nonanisocytic Complexes**

Nonanisocytic complexes consisting of one stoma surrounded by more than three SCs were previously described in Arabidopsis (Yang and Sack, 1995; Serna and Fenoll, 1997; Zhao and Sack, 1999; Geisler et al., 2000; Serna and Fenoll, 2000a). The analysis of nonanisocytic stomatal complexes placed in the borderline of sectors spanning the length of the leaf revealed that at least some of them are polyclonal (Serna and Fenoll, 2000a). Of four stomatal complexes analyzed, one was bisected by the boundary of a sector, indicating that this nonanisocytic complex is polyclonal. The remaining three stomatal complexes were fully stained, and thus cell division patterns that culminate in the formation of both monoclonal and polyclonal complexes can explain their development.

In this work, four sectors staining (totally or partially) these nonanisocytic complexes have been described (Fig. 3A). Two sectors consisted of one stoma surrounded by its four SCs, an indication that these complexes are monoclonal. Two sectors were composed of two SCs belonging to a stomatal complex. Different developmental pathways can explain these partially stained stomatal complexes. For example, two lineages may participate in their formation: one lineage would produce the two stained SCs and the other would form the unstained cells (stoma plus two SCs). Alternatively, they may have a monoclonal origin, based on the cell division pattern represented in Fig. 1 (upper part): A cell division from a SC such that the division plane intersects the stoma would culminate in the formation, from a single precursor cell, of a stoma surrounded by four SCs. This cell division pattern was suggested for the development of nonanisocytic stomatal complexes in other Brassicaceae (Pant and Kidwai, 1967).

Although the total number of nonanisocytic complexes analyzed is very limited (n = 8) due to their low frequency, our data indicate that both monoclonal and polyclonal complexes must be considered.

**A Stereotyped Lineage Accounts for the Stomatal Patterning in Arabidopsis Leaves**

The production of stomata and all their surrounding epidermal cells might in itself create a stomatal spacing pattern. In this work, we show that most stomatal complexes in Arabidopsis (Landsberg erecta ecotype) adaxial leaf epidermis derive from a single precursor cell through a series of oriented divisions that places the stoma towards the center of the stomatal complex (Fig. 1, upper part). In a previous work, we showed that these stomatal complexes exhibit a clustered pattern, that is, most of the stomata are separated by two cells (Serna and Fenoll, 2000a). Taken together the cell division pattern associated with stomatal development and the cell distance between neighboring stomata, it is tempting to conclude that stomatal pattern is achieved through this stereotyped cell division pattern: each stomatal precursor cell gives rise to a entire stomatal complex, consisting of a stoma and all the surrounding SCs, thus, neighboring stomata are placed two cells apart from one another.

A different story seems to take place during the establishment of stomatal pattern in Columbia leaves and cotyledons: all stomatal lineages originate by an unequal division, but the number of subsequent cell divisions is not fixed, and it varies from zero to three (Geisler et al., 2000). As a direct consequence, many stomatal complexes contain at least one nonclonal SCs, indicating that stereotyped divisions can not account for the stomatal pattern (Geisler et al., 2000).

A question that remains to be answered is whether this stereotyped cell division pattern is also sufficient to specify every cell fate during stomatal development. Obviously, the cell lineage-derived mechanism does not exclude the possible release of inhibitory factors from stomata or its precursors during stomatal development or even the consideration of other signaling mechanism (Sachs, 1994; Larkin et al., 1997; Croxdale, 2000). Both cell lineage and cell signaling could be essential for building a stomatal complex.

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**REFERENCES**


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