New Phytologist

Research

Variable timing of developmental progression in the stomatal pathway in *Arabidopsis* cotyledons

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Received: *30 July 2001*
Accepted: *17 October 2001*

Summary

• Stomatal production depends on the rates of precursor cell formation (e.g. the formation of meristemoids by asymmetric division), and of developmental progression (e.g. the division of guard mother cells). It is not known whether these rates follow steady-state kinetics or are variable.

• The timing of development was scored in *Arabidopsis* cotyledons in fixed and living tissue using the dental resin impression method.

• Cotyledons exhibited much less of a longitudinal gradient in stomatal formation than leaves. The timing of the appearance of stomatal and precursor cells during cotyledon development varied between individual plants. Precursor cell formation ceased much earlier in the adaxial than in the abaxial epidermis. Meristemoids are precursors that form guard mother cells. The ratio of these cell types varied greatly in different plants as well as in the same epidermis through time. There was also considerable variability in the duration of the meristemoid stage between individual cell lineages.

• Precursor cell production follows non-steady-state kinetics. Early steps in the pathway are not necessarily synchronized, but later steps, such as the conversion of meristemoids to guard mother cells, sometimes appear to be coordinated.

Key words: *Arabidopsis*, cotyledon, development, epidermis, precursor, stomata, timing.

Introduction

Stomatal number affects the interaction of a plant with its environment, such as the extent of carbon assimilation and transpiration. In turn, environmental factors such as light and CO_2 concentration can regulate stomatal number (Woodward & Kelly, 1995; Boetsch *et al.*, 1996; Lake *et al.*, 2001). Despite the importance of these interactions, little is known about how stomatal number is regulated during development, even when environmental parameters are standardized.

The timing and location of stomatal development differ in monocots and dicots (Sylvester *et al.*, 1996; Croxdale, 2000). Stomatal development in monocots occurs in a strict temporal–spatial gradient in cell files arranged along the length of the leaf (Chin *et al.*, 1995). Although dicots, including *Arabidopsis*, display a roughly longitudinal gradient in young leaves, stomatal development is not rigidly arranged in cell files (Landré, 1972; Lloyd *et al.*, 1994; Donnelly *et al.*, 1999; Geisler *et al.*, 2000). Moreover, during leaf expansion, stomatal precursor cells often form in fields containing mature stomata.

Stomatal development in *Arabidopsis* and other dicots has been studied by the dental resin method, which allows the behavior of the same cells to be followed through time (Kagan *et al.*, 1992; Sachs & Kagan, 1993; Berger & Altmann, 2000; Geisler *et al.*, 2000). In *Arabidopsis*, stomata initiate through an asymmetric division (Fig. 1; Yang & Sack, 1995; Larkin *et al.*, 1997; Geisler *et al.*, 2000). The resulting smaller daughter cell, the meristemoid can also divide asymmetrically. Eventually, the meristemoid converts into the next precursor cell, the guard mother cell (GMC). The GMC divides symmetrically producing the two guard cells of the stoma. Thus,

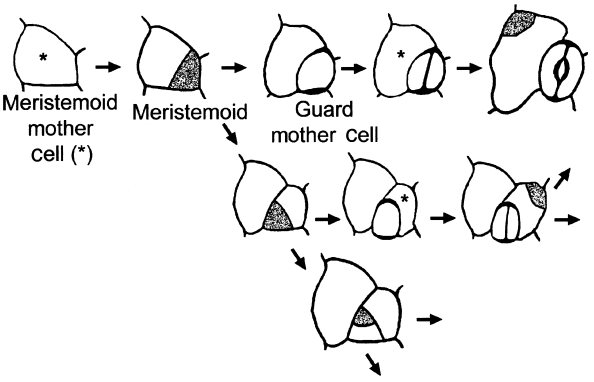

Fig. 1 Stages in stomatal development in *Arabidopsis*. An asymmetric division of the first precursor cell (a meristemoid mother cell, MMC) creates a meristemoid (grey stippling). Meristemoids convert into guard mother cells, with or without first dividing asymmetrically. Guard mother cells divide symmetrically forming two guard cells. When some cells (MMCs marked with an asterisk) reiterate this sequence, the new 'satellite' meristemoid is placed away from the pre-existing stoma or precursor.

stomatal development and cell specification is progressive and involves a series of precursor cells in *Arabidopsis*.

All smaller cells produced by asymmetric divisions in the *Arabidopsis* pathway of stomatal development are meristemoids. The larger daughter cells can themselves divide asymmetrically and form a new meristemoid. As shown in Fig. 1, when these reiterative divisions take place in cells located next to stomata, the divisions are oriented so that the new meristemoid (the satellite meristemoid) is placed away (Larkin *et al.*, 1997; Geisler *et al.*, 2000). Reiterative asymmetric divisions affect stomatal number as well as spacing (Berger & Altmann, 2000; Geisler *et al.*, 2000).

Obviously, precursor cell formation controls stomatal number. But the timing of precursor cell formation at a population level has not been analyzed in *Arabidopsis*. Thus, it is not clear whether precursor cells form and progress developmentally at a uniform rate within the same epidermis through time. Also, little is known about whether these rates vary between the top and bottom epidermis or between different individual plants in a population.

To address these issues, we studied the timing of stomatal development in *Arabidopsis* cotyledons using both fixed and living material. We found that the timing of precursor cell formation differs in the adaxial and abaxial epidermis, and that the balance between formation and developmental progression is frequently nonuniform within an epidermis.

Materials and Methods

Methods for the growth and dental resin analysis of *Arabidopsis thaliana* plants (Columbia ecotype, *gl1* glabrous mutant background) were as described in Geisler *et al.* (2000).

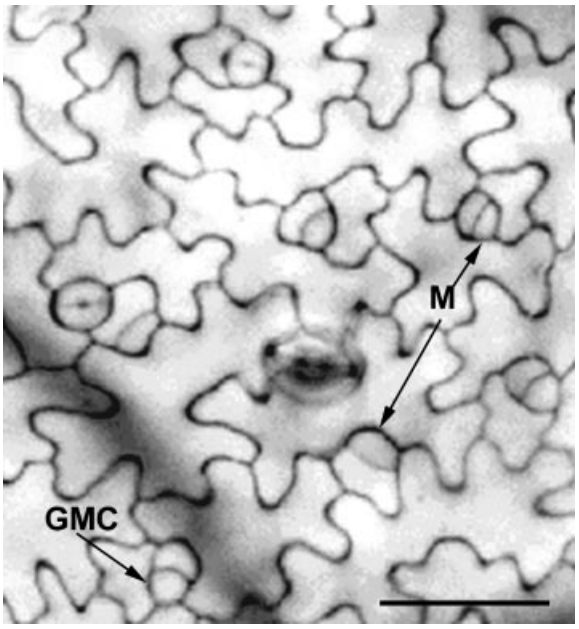

Fig. 2 Light micrograph of abaxial epidermis from cotyledon that was cleared and stained. Meristemoids (M) are more triangular and/or less bulging than mature guard mother cells (GMC). Bar, 25 μm.

Fixed material

Cotyledons were collected at different stages from seeds that were planted at the same time. Seeds were placed on wet germination paper or agar, transferred to darkness at 4°C overnight, and then moved under fluorescent lamps (Geisler *et al.*, 2000). Seedling age was scored starting from when the seeds were placed under the lamps. Seedlings were fixed in 9% formaldehyde, 82% ethyl alcohol, and 9% acetic acid (all v : v). In most cases, the cotyledons were mounted in glycerin and then examined by bright-field microscopy. Other samples were cleared in lactic acid and then stained with safranin, hematoxylin, or fast green. Some cotyledons were dissected from dry seeds that were placed in 50% glycerol.

The frequency of each epidermal cell type was scored from five to 10 randomly selected fields per cotyledon. The combined areas from all fields per cotyledon represented between 10% and 30% of the epidermal surface. Meristemoids were scored based on their small size, their roughly triangular shape and the pattern of surrounding cell walls, indicating an apparent asymmetric division (Fig. 2; Zhao & Sack, 1999). Mature GMCs tend to be larger and have more outwardly curved walls than meristemoids. Because no markers are yet available to distinguish young GMCs from meristemoids, it is likely that the meristemoid sample included some immature GMCs. Newly formed stomata can be readily distinguished from mature GMCs by the presence of a thin dividing wall. All other cells in each field were also counted. To determine the total number of each cell type, means from fields were extrapolated to the entire area of the cotyledon.

Dental resin

Scoring of cell types from impressions used the above criteria as well as data on the behavior of the same cells through time. Because new divisions can only be detected in replicas when the epidermal surface becomes indented, some cells scored as GMCs were probably newly formed stomata. The relative fraction of stomata formed by satellite meristemoids was estimated from dental resin series, as described in Geisler *et al.* (2000). It cannot be ruled out that the impression method itself affects the timing of developmental progression. However, plants subjected to this method appear have normal epidermal cell shape and stomatal patterning.

Results and Discussion

Why cotyledons?

Initially, stomatal formation in *Arabidopsis* follows a more or less tip-to-base gradient in leaves, which means that that stomata first form at the leaf tip and then gradually appear lower down (Pyke *et al.*, 1991; Donnelly *et al.*, 1999). This gradient complicates population level studies of the timing of stomatal development. However, this gradient is reduced or absent in cotyledons (Fig. 3). For this reason, cotyledons were used for timing studies. The difference in the extent of this gradient in cotyledons and leaves might be due to the smaller final size of cotyledons, or to differences in embryonic and post-embryonic programs (Wei *et al.*, 1994).

Variable timing of development in different plants

Using fixed material, we first analyzed the relationship between indicators of cotyledon development and stomatal frequency. Parameters measured included cotyledon length, paradermal area, age and total cell number in the adaxial or abaxial epidermis. Cotyledon age appeared to be a secondary indicator of stomatal number, especially in the adaxial epidermis. The best indicator was total cell number, which correlated strongly with stomatal number in the abaxial epidermis (correlation coefficient of 0.9, Table 1; Fig. 4). The correlation between epidermal cell and stomatal number was lower for the adaxial epidermis ($R = 0.6$ for Table 2). Some cotyledons with the same total number of adaxial epidermal cells had dramatically different numbers of stomata (Table 2). For example, a 36-h cotyledon with an estimated 846 epidermal cells had only 10 stomata, whereas a 192-h cotyledon with 834 cells had 167 stomata. Thus, while these indicators help predict stomatal number, there is still substantial variation between different plants of the same age or total cell number.

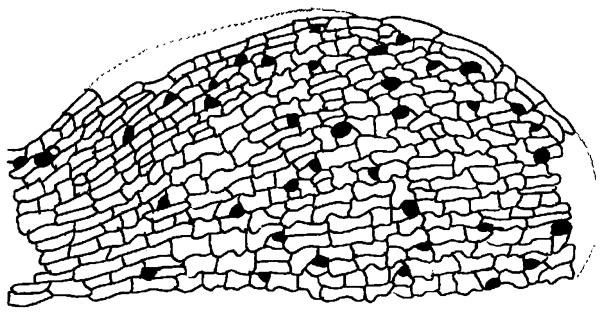

Fig. 3 Stomatal precursors are scattered throughout a developing cotyledon. Tracing from abaxial epidermis of fixed cotyledon from ungerminated seed. Meristemoids and guard mother cells are shown in black. No stomata were yet present. Bar, 100 μm.

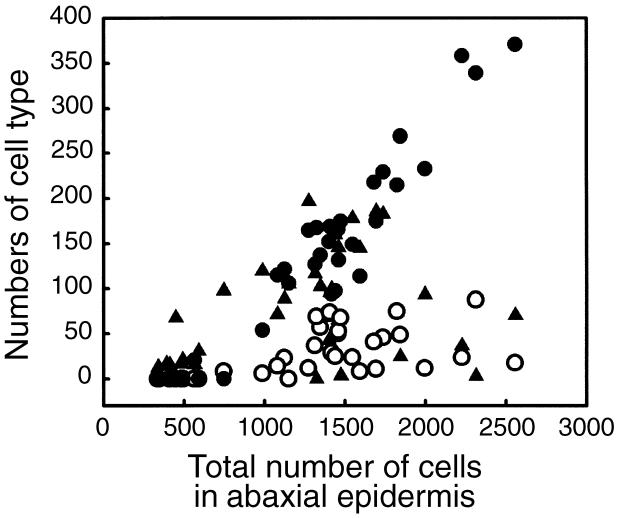

Fig. 4 Number of stomata and precursor cells in the abaxial epidermis as a function of total cell number in different cotyledons fixed at different ages; triangles, meristemoids; open circles, guard mother cells; closed circles, stomata; $n = 100$ cotyledons.

This variability extended to the early stages of cotyledon development, including during the formation of the seed. Landré (1972) found that cotyledons of an *Arabidopsis* relative, *Sinapsis alba*, lacked stomata in dry seeds but did have stomatal precursors. We occasionally found stomata in cotyledons from dry *Arabidopsis* seeds, but usually only precursors were present and stomata did not appear until day 2 (Tables 1 and 2). There was considerable variability in the number of stomatal precursors present during days 0–2. Some cotyledons from dry seeds had the same number of precursor cells as those from 36 h plants. These trends were found in both sides of the cotyledon (Tables 1 and 2). These data highlight the non-uniformity in the numbers and timing of precursor cells and stomata from plant to plant.

Adaxial epidermis exits stomatal development earlier

The cotyledon abaxial epidermis has about five times as many stomata as the adaxial side (Geisler *et al.*, 1998). This could result from different rates of precursor cell formation, or from comparable rates for different periods. We found that stomatal precursor cell formation stops much earlier in the

Table 1 Cell numbers in the abaxial epidermis of cotyledons that were fixed at different ages

Age when fixed (h)	Cotyledon length (mm)	Cotyledon area (mm^2)	Total cell number	Meristemoids	Guard mother cells	Stomata
0	0.20	0.05	343	13	0	0
0	0.38	0.09	747	98	8	0
12	0.43	0.10	485	17	0	0
12	0.46	0.12	339	13	0	0
12	0.52	0.14	392	17	3	0
15	0.43	0.11	493	21	0	0
15	0.44	0.12	410	16	0	0
18	0.41	0.10	592	31	0	0
18	0.46	0.13	449	68	0	0
21	0.46	0.13	566	16	0	21
39	0.61	0.20	1152	106	0	106
127	0.86	0.57	987	120	6	54
127	0.73	0.39	1738	183	46	229
144	1.17	0.99	1125	89	23	122
144	1.02	0.79	1696	186	11	175
144	1.17	0.94	1080	72	14	115
144	0.91	0.62	1315	117	37	127
168	1.02	0.79	1547	178	24	149
168	1.00	0.79	1824	75	75	215
192	1.12	1.01	1349	103	57	137
192	1.17	0.99	1401	43	73	152
			1276	197	12	165
			1325	0	69	168
			1409	96	74	169
			1418	100	29	94
			1440	160	25	98
			1459	66	50	166
			1460	146	53	132
200–264			1476	4	68	175
			1595	145	8	114
			1680	41	41	218
			1844	25	49	269
			1998	94	12	233
			2227	37	24	358
			2315	3	88	339
			2559	71	18	371

Age represents time (h) after imbibed seeds were exposed to light. With the exception of stomata and guard mother cells from very young cotyledons (up to 21 h), all numbers for cell types are estimates based on extrapolations from three fields per cotyledon. The bottom part of the Table is from 8 to 11-day-old-cotyledons and is sorted by total cell number. The 'Total Cell Number' column includes all epidermal cells, such as pavement cells, in addition to those cell types shown in the table.

adaxial than abaxial epidermis (Tables 1 and 2; Fig. 5). For example, no precursor cells (meristemoids or GMCs) were present in the adaxial epidermis in many cotyledons older than 127 h, whereas in the abaxial epidermis precursors were present in older cotyledons (Tables 1 and 2). Formation of GMCs stops when the adaxial epidermis has many fewer cells than the abaxial side (Fig. 5). This halt in GMC formation occurs when cotyledons are still expanding (data not shown). As a result, pavement cells tend to be larger in the adaxial than abaxial epidermis.

The abaxial and adaxial epidermises also differ in the fraction of stomata produced by satellite meristemoids. About 75% of abaxial stomata in cotyledons derive from satellite meristemoids (Geisler *et al.*, 2000), whereas only about 35% do so in the adaxial epidermis. A similar trend was found in mustard (Landré, 1972). This difference between the adaxial and abaxial epidermis is expected because, by definition, satellite meristemoids form in cells next to stomata (or precursors). Members of the Brassicaceae can have up to four generations of satellite meristemoids with each new satellite originating in a neighbor cell produced by previous asymmetric divisions (Landré, 1972; Berger & Altmann, 2000). Because stomatal precursor cell formation stops sooner in the adaxial than abaxial epidermis, the adaxial side would have fewer stomatal complexes and neighbor cells that could form satellite meristemoids.

The more fundamental question is the identity of the signal that terminates stomatal precursor cell formation earlier in the

Table 2 Cell numbers in the adaxial epidermis of cotyledons that were fixed at different ages

Age when fixed (h)	Cotyledon length (mm)	Cotyledon area (mm^2)	Total cell number	Meristemoids	Guard mother cells	Stomata
0	0.34	0.07	315	11	0	0
12	0.30	0.09	490	29	9	2
12	0.39	0.10	356	12	0	0
12	0.46	0.13	598	15	10	0
15	0.47	0.12	508	19	0	0
15	0.51	0.13	470	29	0	0
15	0.43	0.11	418	18	3	0
18	0.46	0.13	422	8	0	0
18	0.43	0.12	536	19	6	0
18	0.42	0.10	380	9	0	0
24	0.36	0.09	402	12	17	0
36	0.48	0.13	497	15	26	0
36	0.57	0.18	607	49	20	0
36	0.68	0.23	846	34	34	10
36	0.61	0.18	578	14	19	33
127	0.95	0.75	968	0	17	138
144	1.00	0.80	707	9	0	102
144	0.98	0.66	745	0	15	137
168	1.10	0.97	776	15	15	142
168	1.10	0.88	704	0	0	131
168	1.00	0.71	782	13	0	131
192	1.10	0.86	679	0	0	146
192	1.17	1.03	834	0	0	167
192	1.20	1.10	674	0	0	140

Age represents time (h) after imbibed seeds were exposed to light. With the exception of stomata and guard mother cells from very young cotyledons (up to 24 h), all numbers for cell types are estimates based on extrapolations from three fields per cotyledon. The 'Total cell number' column includes all epidermal cells, such as pavement cells, in addition to those cell types shown in the table.

adaxial side. Although not quantified, all types of divisions appeared to stop in the adaxial epidermis when GMC formation ceased. Thus the signal that halts stomatal formation in the adaxial epidermis could be one that generally affects division competence such as the induction of endoreduplication (Melaragno *et al.*, 1993).

The increase in the number of epidermal cells through time in both sides of the cotyledon contrasts with the situation inside this organ. During cotyledon development, there is little cell division in the mesophyll, and most growth results from cell expansion (Tsukaya *et al.*, 1994).

Nonuniform developmental progression

We next studied whether the rates of precursor cell formation and developmental progression are uniform in the abaxial epidermis at different stages of cotyledon growth. In principle, the numbers of each cell type at any point in time depend upon where that cell type is located in the pathway. For example, meristemoid number depends on how many meristemoids are produced, on how many times meristemoids divide, and on how long the meristemoid takes to convert into a GMC. Similarly, stomatal number depends on the number of precursor cells formed and on how long it takes for GMCs to

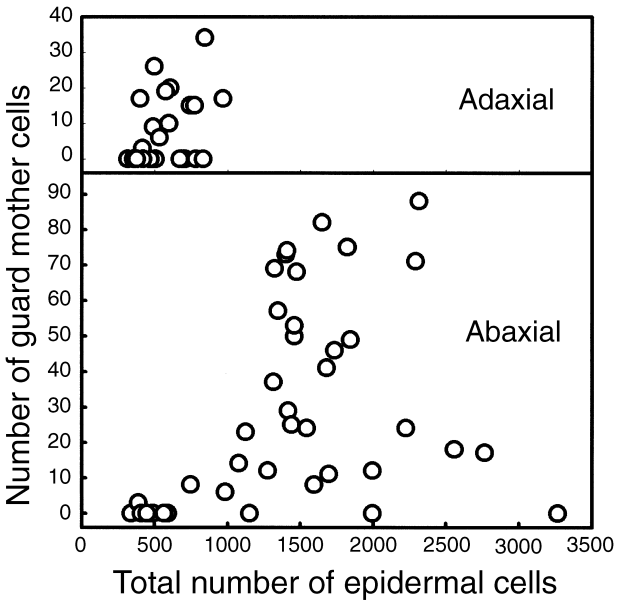

Fig. 5 Number of guard mother cells per cotyledon as a function of the total number of epidermal cells. Guard mother cell formation stops on the adaxial side when that epidermis contains many fewer cells than ultimately form in the abaxial epidermis. Cotyledons were chemically fixed and both sides scored. Numbers are extrapolations.

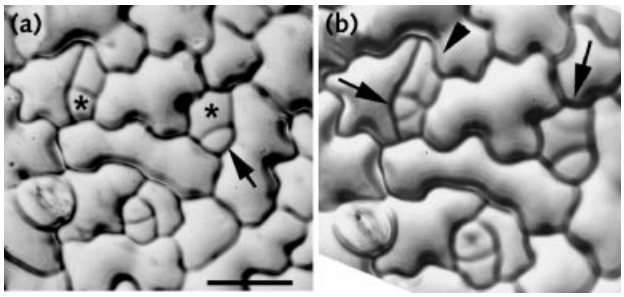

Fig. 6 Dental resin series. Bright-field light micrographs of nail-polish replicas of dental resin impressions showing the same field of cells over a 2-day interval. (a) The asterisks indicate two cells that divide asymmetrically within 24 h. (b) Division of the left cell, which is a meristemoid, regenerates the meristemoid (left arrow in b). The right cell in (a) that is marked with an asterisk is a meristemoid mother cell (MMC) that is located next to a meristemoid (indicated by arrow); division of this MMC produces a correctly placed satellite meristemoid (right arrow in b). Note symmetric division (arrowhead in b). Bar, 25 μm.

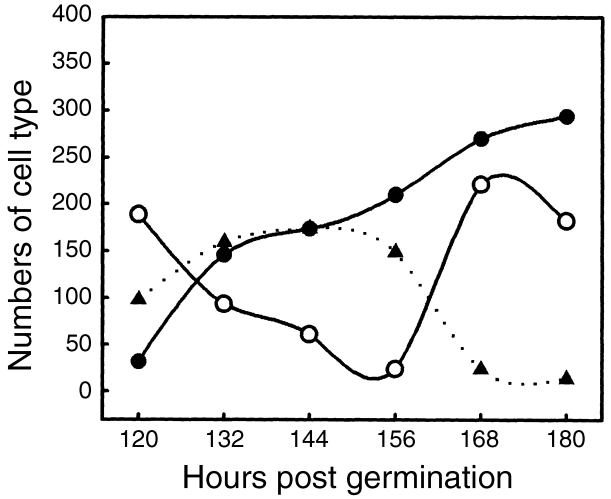

Fig. 7 Coordinated developmental progression of precursor cells in the same epidermis through time. Six successive peels were taken from the same abaxial region of the same cotyledon every 12 h starting 5 days post germination: triangles, meristemoids; open circles, guard mother cells; closed circles, stomata. Spline curves fitted to data.

divide, and so on. It is possible, for example, that the rate of production of new meristemoids matches the rates of conversion of meristemoids to GMCs and of GMCs to stomata. This could produce steady-state levels in the ratios of different precursor cells to each other and to stomata.

To evaluate whether there is uniformity in the ratios of precursor cells, we analyzed their relative frequencies in fixed tissue. Typically, young cotyledons with fewer than 1000 cells had few or no GMCs (Table 1). This is expected because meristemoids are the first detectable precursor cell to form and because GMCs develop from meristemoids. The older (200–264 h) cotyledons that we sampled were still developing, as shown by the presence of precursor cells. In older cotyledons with more total cells there was considerable variability in the relative frequencies of meristemoids and GMCs. Some cotyledons had almost no meristemoids and many mature GMCs, while in other cotyledons the reverse was true. These variations did not simply correlate with relative age or total cell number. The presence of many more GMCs than meristemoids might signal the onset of epidermal maturation. Alternatively, these variations could occur if precursor cells were produced or matured in pulses or waves.

Because it is difficult to evaluate these alternatives using destructively sampled tissue, we studied the behavior of cells in the same epidermis through time using the dental resin method. Figure 6 shows an example of the resolution and information obtained using this technique. As indicated (see the Methods and Materials section), it is not possible to distinguish between mature GMCs and very young stomata using dental resin replicas. Nevertheless, the GMC category is a defined developmental window whose timing can be scored. We first calculated the number of different cell types found through time in single dental resin series (Fig. 7). The number of GMCs decreased and the number stomata detected increased between 120 h and 156 h, suggesting that many of the mature GMCs divided and produced stomata. Later (156–168 h), the number of meristemoids decreased and the number of mature GMCs increased, suggesting that at least some meristemoids converted into GMCs during this 12 h period. These data suggest that the timing of developmental progression through the stomatal pathway is not uniform.

To explore this further, dental resin series were taken from the abaxial epidermis of four different cotyledons during overlapping periods that spanned 2–13 d (Table 3). The number of stomata rose with age, cotyledon area, and total cell number. The number of cells scored as GMCs and meristemoids was not constant throughout development. Instead, there were 12-h periods when the number of GMCs or meristemoids rose or dropped significantly more than in other periods (Table 3). For example, between 120 h and 132 h (see Table 3, third series), the number of meristemoids and GMCs slightly increased and decreased, respectively. In the next 12-h period (132–144 h), the number of meristemoids dropped from 204 to 103, while GMCs increased from 4 to 123. In the next 12-h period (144–156 h), the number of meristemoids and GMCs increased by 15–19%. These data are inconsistent with a steady-state hypothesis, and instead suggest that there are pulses in the rates of precursor cell formation during the growth of cotyledons. For example, a cohort of meristemoids might form, divide and convert into GMCs at the same time resulting in a burst in the number of stomata formed.

To address this possibility at a finer scale, the timing of the developmental progression of different meristemoids was scored. Figure 8 shows data from one dental resin series (seven peels, six of them 12 h apart) from two different areas (apex vs base) of the same cotyledon. Considerable variation was found in the duration of different precursor cell stages. Some

Table 3 Changes in cell numbers in the abaxial epidermis during cotyledon development

Age (h)	Cotyledon area (mm^2)	Total cells	Meristemoids	GMCs	Stomata
48	0.27	970	75	10	39
60	0.33	1090	137	16	46
72	0.6	1130	124	***5***[1]	64
84	0.65	1200	54	***74***	74
96	0.74	1280	64	53	83
96	0.61	920	28	32	93
108	0.67	1110	20	61	117
120	0.82	1280	37	***7***[1]	153
132	0.96	1520	66	***66***	168
228	3.36	2350	68	9	384
272	4.39	2390	11[1]	67[1]	401
120	1.41	1730	197	13	160
132	1.43	1860	204	***4***[1]	180
144	1.8	2160	103	***123***	210
156	2.28	2280	123	142	235
168	2.68	2490	80	88	265
180	2.87	2600	55	33	380
240	5.39	2960	68	55	545
288	5.44	3060	93[1]	10[1]	590
312	5.98	3090	10[1]	81[1]	590
168	1.19	2009	146	36	170
216	1.99	2288	57	81	250
312	3.94	2931	64	***0***[1]	435
456	5.48	3306	18	***71***	462
504	6.43	3922	52	17	543
672	7.80	4448	0[1]	0[1]	569

The data were obtained from dental resin impressions from four different cotyledons (thick lines separate each series). The pairs of numbers in bold italic in the guard mother cells (GMCs) column identify a relatively large rise over a 12-h period. Most numbers shown are extrapolations from impressions that together covered about 10–30% of the area of the entire cotyledon. [1]In selected cases, the number of meristemoids or GMCs in the entire cotyledon was counted; this was done when possible (impressions were available that covered the entire cotyledon) and when there were relatively large changes in numbers between impressions.

meristemoids persisted as long as 48 h. Other meristemoids divided or developed into mature GMCs within 12 h of their formation. There were several periods (132–144 h and 156–168 h) when the majority of meristemoids became mature GMCs, even though these meristemoids had arisen at different times and divided a variable number of times. Similar trends were found in dental resin series from two other cotyledons (data not shown). Although limited in sample size, these data raise the possibility that there may be some coordination in the timing of when meristemoids convert into GMCs. In addition, our data argue against the idea that a cohort group of meristemoids forms at the same time and progresses through subsequent stages coordinately.

Collectively, these data show that the rates of production and the lifespans of stomatal precursors are variable during cotyledon development in *Arabidopsis*. They also hint that the timing of some developmental progressions may be coordinated across an epidermis. It has been reported that mature stomata appear in waves in some dicotyledon species (Leick, 1955). As markers for different stages of precursor cell development become available, it should be possible to expand this analysis using nondestructive imaging and larger sample sizes. In monocots, stomatal fate may be specified coordinately in groups of cells that are at the same stage of the cell cycle, and precursor cell formation has been shown to follow diurnal rhythms (Zeiger & Cardemil, 1973; Chin *et al.*, 1995; Croxdale, 2000).

Our data show that the regulation of the timing of precursor cell formation and of developmental progression can affect stomatal number in *Arabidopsis*. Examples include differences between the abaxial and adaxial cotyledon epidermis as well as apparent pulses in precursor cell formation during the development of an individual epidermis. A key question for future research is how this timing is regulated and whether it responds to factors such as circadian rhythms, the stage of the cell cycle, the photoperiod, and tissue level signals. The identification of factors controlling stomatal number is important for understanding leaf development and plant productivity.

Acknowledgements

This research was supported by grants from the Developmental Mechanisms Program of the National Science Foundation

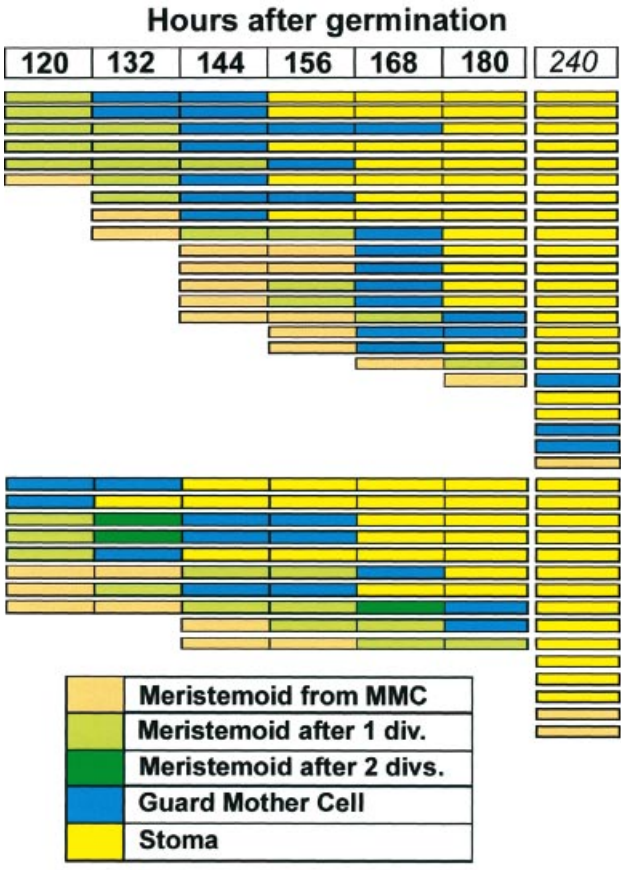

Fig. 8 Graph from dental impressions showing that the lifespans of meristemoids and guard mother cells are variable. Each horizontal bar represents one stomatal lineage whose stages are color-coded. For example, meristemoids can arise from an asymmetric division of a meristemoid mother cell, or can be regenerated when meristemoids divide once or twice. The series were taken from two sample areas of the abaxial epidermis from the same cotyledon. New meristemoids arose in every peel (indicated by new bars). Many meristemoids converted to guard mother cells in two intervals (132–144 and 156–168 h).

(nos IBN-9505687 and IBN-9904826). Jeanette Nadeau is thanked for numerous helpful discussions, and Connie Lee and Jochen Schwuchow are thanked for technical assistance.

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