

# The *too many mouths* and *four lips* Mutations Affect Stomatal Production in Arabidopsis

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**Stomata regulate gas exchange through the aerial plant epidermis by controlling the width of a pore bordered by two guard cells. Little is known about the genes that regulate stomatal development. We screened cotyledons from ethyl methanesulfonate-mutagenized seeds of Arabidopsis by light microscopy to identify mutants with altered stomatal morphology. Two mutants, designated *too many mouths* (*tmm*) and *four lips* (*flp*), were isolated with extra adjacent stomata. The *tmm* mutation results in stomatal clustering and increased precursor cell formation in cotyledons and a virtual absence of stomata in the inflorescence stem. The *flp* mutation results in many paired stomata and a small percentage of unpaired guard cells in cotyledons. The double mutant (*tmm flp*) exhibits aspects of both parental phenotypes. Both mutations appear to affect stomatal production more than patterning or differentiation. *tmm* regulates stomatal production by controlling the formation, and probably the activity, of the stomatal precursor cell.**

## INTRODUCTION

Stomata are essential for plant productivity and were a crucial adaptation during the evolution of land plants. They control gas exchange for photosynthesis by adjusting the width of a pore flanked by two guard cells (Raschke, 1979). Stomata are found on most aerial surfaces of leaves, stems, and reproductive parts and are usually distributed in a characteristic pattern. Despite their importance and well-studied differentiation (Sack, 1987), little is known about the mechanisms that underlie stomatal patterning, determination, and differentiation, and virtually nothing is known about the genes that regulate these processes.

In many plants, the pattern and distribution of stomata are established by the formation of meristemoids, cells that continue to divide after most surrounding cells have stopped cycling (Bünning, 1953). In the Brassicaceae, a family that includes Arabidopsis, stomatal development depends on meristemoid formation and activity (Paliwal, 1967; Pant and Kidwai, 1967; Landré, 1972). As shown in Figure 1, the meristemoid gives rise to a guard mother cell but first produces other epidermal cells (ECs). Thus, the meristemoid is the precursor cell of both the guard cells and the adjacent ECs, and in this article, the terms "precursor cell" and "meristemoid" are used synonymously.

Stomatal placement is not random in dicots; the occurrence of adjacent stomata is much rarer than would be the case in a random distribution. In fact, one of the most ordered features of stomatal spacing is the presence of a stoma-free region surrounding each stoma (Sachs, 1991). In many plants, the

production of epidermal cells from the meristemoid apparently ensures that in wild-type plants, mature stomata are not in contact with one another.

Mutations would be valuable for understanding stomatal development, but the only structural mutation reported to date for stomata is *eceriferum-g* of barley, which has double and triple adjacent stomatal complexes (Zeiger and Stebbins, 1972). Thus, we are largely ignorant of the genes controlling meristemoid formation and activity and guard cell differentiation. In contrast, more than 30 different loci that affect the cell development of trichomes or root hairs in Arabidopsis have been identified by mutation (Schiefelbein and Somerville, 1990; Oppenheimer et al., 1992; Hülskamp et al., 1994; Masucci and Schiefelbein, 1994), and several of these genes have been cloned (Herman and Marks, 1989; Rerie et al., 1994).

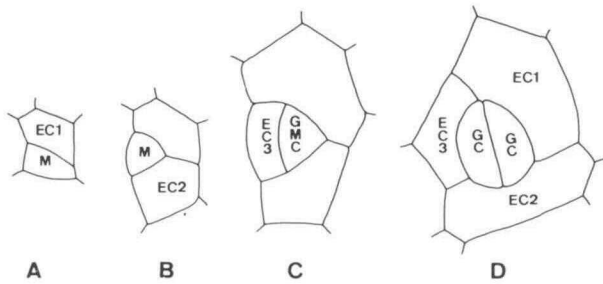
To better understand how stomata and guard cells develop, we attempted to isolate mutations that affect stomatal morphology and distribution in Arabidopsis. Here, we describe two mutations, *too many mouths* (*tmm*) and *four lips* (*flp*), that have extra adjacent stomata. Both mutations influence the production of stomata, and *tmm* also regulates meristemoid formation.

## RESULTS

### Stomata in Wild-Type Cotyledons

Arabidopsis cotyledons are small, leaflike photosynthetic organs initiated during embryogenesis. Expanded cotyledons

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**Figure 1.** Stomatal Development in the Brassicaceae.

Stomatal precursor cell (meristemoid) formation and activity as described for various genera in the Brassicaceae. Sequence is redrawn from Landré (1972) and is based on inferences from cell wall and cell division patterns, not from the study of the same cells through time. (A) A stomatal meristemoid forms through the asymmetric division of a protodermal cell. The larger cell product becomes a nonstomatal EC, and the smaller cell becomes a meristemoid (M).

(B) and (C) The meristemoid then undergoes one to several asymmetric divisions. After each division, the larger cell product differentiates into an EC. The smaller daughter cell continues to act as a meristemoid and divides asymmetrically. The numbering of ECs indicates the order of their formation. After the last EC is produced, the meristemoid converts into a guard mother cell (GMC).

(D) The guard mother cell then undergoes a terminal symmetric division that produces two guard cells (GCs; stomatal pore not yet formed in [D]).

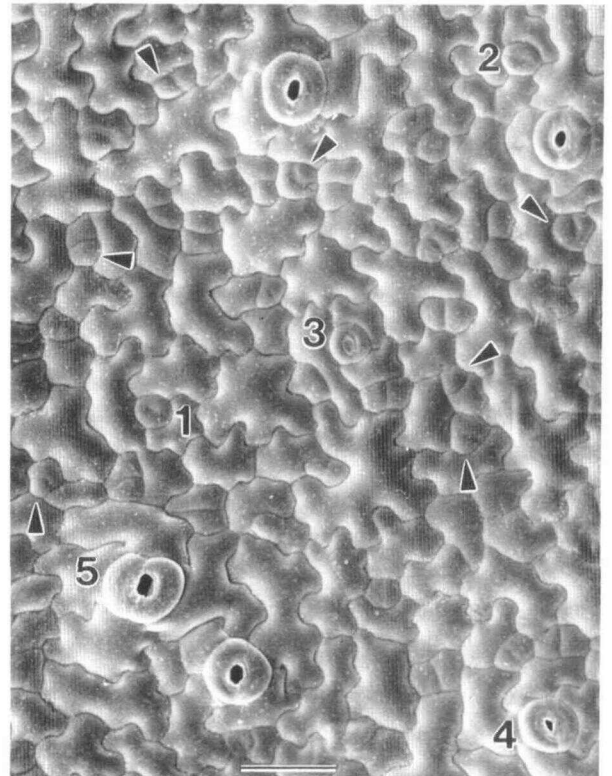
contain many mature and apparently functional stomata, especially in the abaxial epidermis. Cotyledons were the primary organ used in screens for stomatal mutations (see Methods). The abaxial epidermis of the cotyledon was screened from seedlings that were 12 days old or older, when the cotyledons had stopped expanding in area. This report focuses on cotyledons, in part because the *tmm* phenotype is more strongly expressed in cotyledons than in leaves (see below; M.J. Geisler and F.D. Sack, unpublished data).

In wild-type cotyledons, stomata are separated by one or more nonstomatal epidermal cells (Figure 2). Most, but not all, epidermal cells adjoin guard cells. As in most crucifers, many stomatal complexes in the *Arabidopsis* cotyledon are anisocytic, that is, the stomata are surrounded by three epidermal cells (subsidiary cells), one of which is smaller (Figures 1D and 2; Telfer and Poethig, 1994). However, many other stomatal complexes lack this configuration, and instead the stomata are surrounded by four or five epidermal cells with no smaller epidermal cells (Figure 2). Because the number, arrangement, and size of epidermal cells around stomata vary, subsidiary cells, in the sense of morphologically distinguishable cells adjacent to stomata (Esau, 1977), are not universally present in *Arabidopsis*; thus, the more inclusive term "epidermal cell" is used here instead of "subsidiary cell" to describe the cells that neighbor guard cells.

Meristemoids can be identified as small cells next to somewhat larger cells, with the alignment of walls indicating that

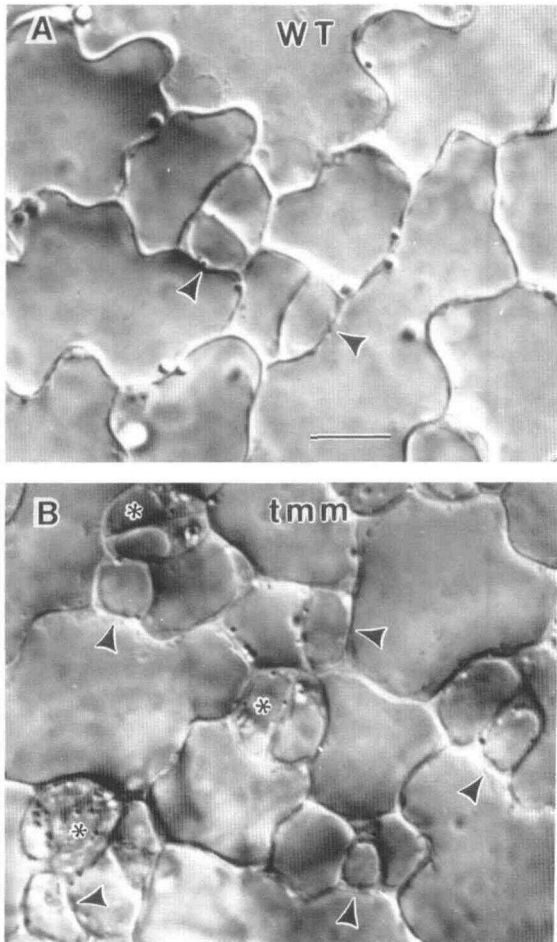
both cells were produced by an earlier asymmetrical division (Figures 1 to 3). Meristemoids are numerous in developing cotyledons and appear to have a distribution (patterning) and density related to those of stomata (Figure 2). Cell patterns similar to those depicted in Figure 1 were frequently found (Figure 3). Meristemoids are also present in regions containing mature stomata (Figure 2), even in fully expanded cotyledons.

In wild-type plants, mature guard cells are kidney shaped, and the stomatal pore is lenticular (Figures 4A and 5A). Several stages of pore development were identified using cryoscanning electron microscopy (Figure 2). First, a small, median, slightly elongated depression forms along the long axis of the cell. This depression extends and then develops into an elongated, lens-shaped plug that presumably consists of wax and cutin. This stage is followed by pore opening and guard cell maturation.



**Figure 2.** Stomatal Development in a Wild-Type *Arabidopsis* Cotyledon.

This cryoscanning electron micrograph shows that stomatal development is asynchronous. The smaller cells are meristemoids (arrowheads) and guard mother cells. Consecutive stages of stomatal differentiation are numbered. Pore wall formation is first detectable as a central indentation (1), which then extends to the full length of the developing stoma (2). A lenticular wax plug then develops (3), pore formation (i.e., separation) starts (4), and then extends to the final length (5). Note the successively increasing cell size from meristemoid to stoma. Note as well that the number of ECs neighboring each mature stoma varies. Bar = 30  $\mu$ m.



**Figure 3.** Meristemoids in Developing Cotyledons.

These light micrographs show the abaxial epidermis of 4-day-old cotyledons. Fresh tissue was sectioned by hand and viewed with differential interference contrast optics.

(A) Wild type (WT) showing two meristemoids (arrowheads). Although not shown here, maturing stomata were frequently found at this stage. (B) *tmm* showing meristemoids (arrowheads), some of which are located adjacent to developing stomata (asterisks). Note the higher density of meristemoids in *tmm* compared with the wild type.

Bar in (A) = 10  $\mu$ m for (A) and (B).

### too many mouths

The mutant *tmm* has clustered stomata (Figures 4C, 5B, and 6A). The word "stoma" is derived from the Greek for "mouth," and so, by analogy, the guard cells are the "lips."

To facilitate the description of mutant phenotypes, it was useful to create a new term, the stomatal unit (SU), which is defined here as one or more guard cells in contact with each other. Thus, in the wild type, in almost all cases, a stomatal unit and a stoma are synonymous, and of course each stomatal unit

contains two guard cells. However, in *tmm*, ~55% of the SUs contain more than one stoma, that is, the SUs consist of clustered stomata. These clusters contain up to 26 guard cells (Figure 7). The remaining 45% of the SUs in *tmm* consist of only two guard cells and resemble wild-type stomata. In *tmm*, SUs have only an even number of guard cells (Figure 7). Approximately three out of four SUs that are clustered contain no more than eight guard cells.

In developing or even fully expanded cotyledons, the stomata within a cluster are often at different stages of development, and the stomata are arranged at many angles with respect to each other so that the long axes of their pores are rarely parallel (Figures 4C, 5B, and 6A). Clusters vary in shape from compact groups to small spirals or irregular patterns.

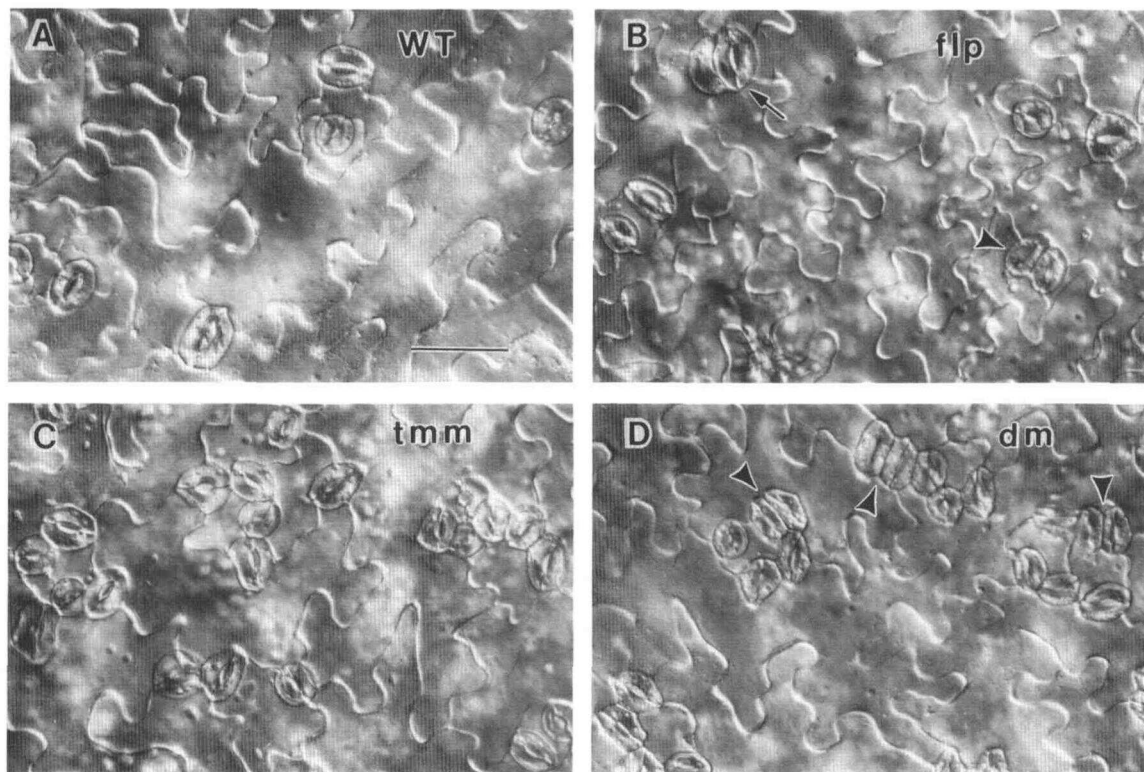
Stomatal pore formation is arrested developmentally in some stomata in clusters, even from fully expanded cotyledons. Arrest appears to occur after depression and plug formation so that opening never occurs (Figure 5B). Other stomata in clusters have open pores smaller than those of the wild type. Stomata with arrested pores appear smaller and more constrained in cell expansion. Larger stomata in clusters usually have pores of normal size and shape. Guard cell shape is often normal in *tmm*, but abnormal shapes also occur, particularly in stomata whose cell expansion appears restricted due to cell packing.

Developing *tmm* cotyledons were examined to detect earlier stages in cluster formation. In *tmm*, meristemoids are often found adjacent to developing stomata and guard mother cells (Figure 3B). These cells can be identified as meristemoids because they appear to be the smaller product of an unequal division and because they resemble wild-type meristemoids in size and shape (Figures 3A and 3B). In wild-type cotyledons of the same age, meristemoids were never seen in contact with developing stomata or guard mother cells. The presence of a meristemoid next to a differentiating stoma in young *tmm* cotyledons is consistent with the observation that stomata in mature clusters are often found in different stages of development.

The presence of stomatal clustering indicates that the *tmm* mutation increases the number of guard mother cells produced. The fact that these extra stomata form next to other stomata also shows that the *tmm* mutation can prevent the formation of a stoma-free zone characteristic of the wild type. Although abnormal pore development and guard cell shape can be found in *tmm*, particularly where stomatal crowding is induced, the primary effect of this mutation appears to be on stomatal production (stomatal number) rather than on differentiation.

### four lips

The *flp* phenotype is characterized by the presence of two adjacent stomata (Figures 4B, 5C, 7, and 8A). Another distinctive trait of *flp* is the presence of a small percentage of unpaired guard cells, either single and isolated or part of a small cluster



**Figure 4.** Light Microscopy of the Wild-Type and Stomatal Mutants.

The abaxial epidermis of cotyledons is shown. Extra adjacent stomata are present in all three mutant genotypes. Whole mounts were cleared in ethanol and viewed with differential interference contrast optics (out-of-focus mesophyll starch is visible in some regions).

(A) Wild type (WT). Stomata are single and separated from each other by at least one EC.

(B) *flp*. Abnormal stomatal units include a pair of adjacent stomata (arrowhead) and a group of three guard cells consisting of an unpaired guard cell adjacent to a stoma (arrow).

(C) *tmm*. The clusters of stomata are arranged in various patterns but not laterally.

(D) Double mutant (*dm*). Stomatal clusters include some laterally arranged stomata (arrowheads).

Bar in (A) = 40  $\mu$ m for (A) to (D).

(Figures 4B and 8B to 8D). Thus, *flp*, unlike *tmm*, has SUs with either an even or an odd number of guard cells (Figure 7). Of all SUs, 21% consisted of four guard cells per unit and ~8% consisted of one, three, five, six, seven, or eight guard cells per unit. Altogether, ~29% of all SUs were abnormal and 71% were similar to wild-type stomata.

In some clusters, adjacent stomata were positioned side by side, that is, laterally (Figures 4B, 5C, and 8A). In these cases, the stomatal pores were often parallel. Differentiation was usually normal even in unpaired guard cells (Figures 8B to 8D). However, pore development sometimes appeared arrested, and some unpaired guard cells were abnormally shaped.

The stomatal clusters of *flp*, as well as those of *tmm*, seem to be distributed in a pattern reminiscent of the wild type, with each cluster or single guard cell positionally equivalent to a single stoma in the wild type (Figures 4A to 4C and 5A to 5C).

Thus, like *tmm*, *flp* appears to affect primarily the production of guard mother cells rather than meristemoid patterning or stomatal differentiation. However, these two phenotypes differ in that *flp* clusters are smaller and have a different morphology than *tmm*, and of the two, only *flp* has unpaired guard cells.

Genetic analysis indicates that *flp* and *tmm* are separate loci because crosses between mutants yielded  $F_1$  plants with a wild-type stomatal phenotype (Table 1). They are also nuclear recessive; approximately one of four  $F_2$  plants showed (following separate backcrosses of each mutant to the wild type) the single mutant phenotype. It is possible that both loci are weakly linked, because the low  $P$  value ( $>0.10$ ) indicates that the observed segregation ratio ( $F_2$  plants following the cross of *flp* and *tmm*) does not conform strongly to a ratio of 9:3:3:1. After several backcrosses, each mutant phenotype resembled that



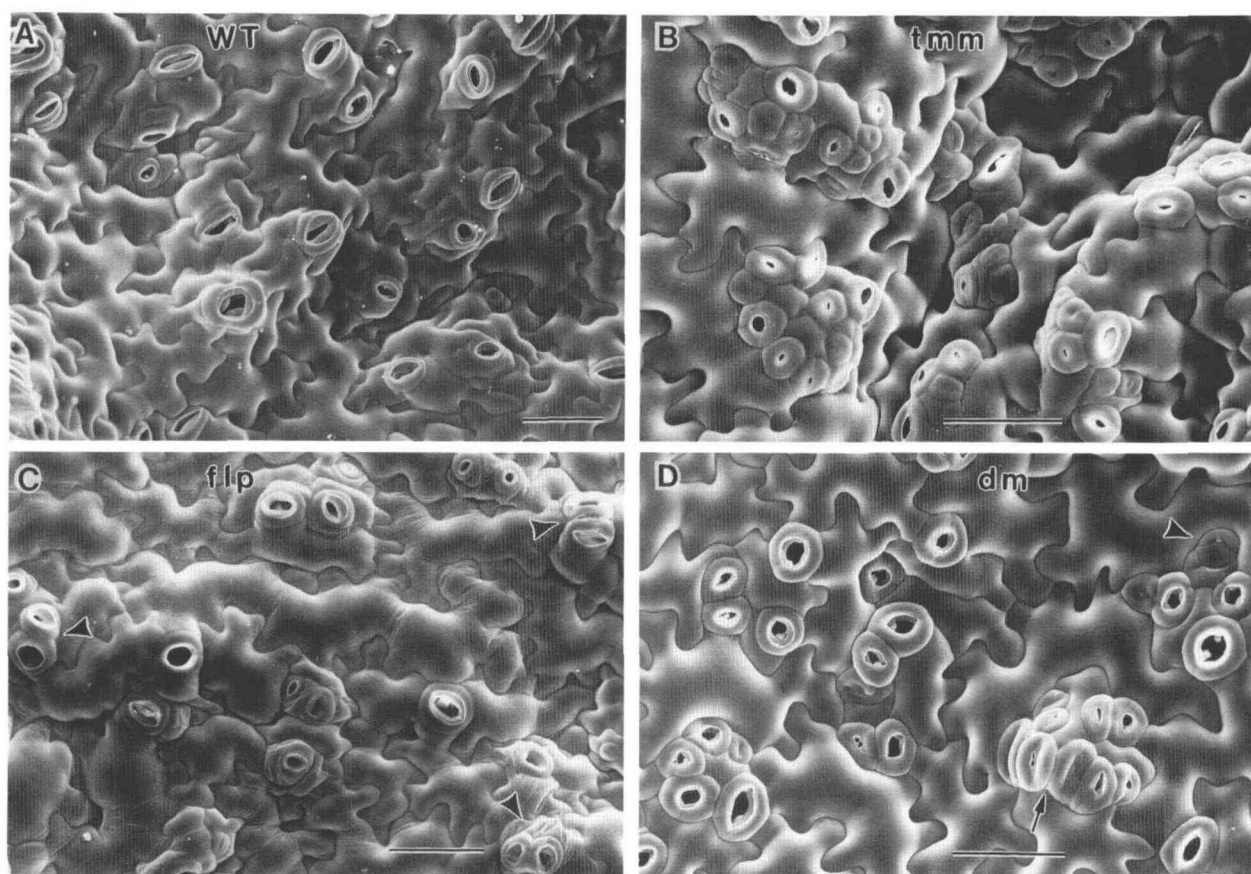
of the original  $M_2$  isolate, indicating that it is unlikely that secondary mutations are responsible for the phenotypes described. Only one allele has been found for each locus, *tmm-1* and *flp-1*.

### Double Mutant

Following a dihybrid cross, the double mutant was identified by the presence of a new phenotype characterized by more and larger clusters than *flp*, with SUs containing even and odd numbers of guard cells (Figures 4D, 5D, and 6B).

After self-fertilization, this new phenotype was stable and present in all progeny for five generations. These presumptive double mutant plants were then backcrossed separately to *tmm* or *flp* (at least six independent crosses per mutant parent). All  $F_1$  plants ( $\geq 82$ ) from each set of crosses displayed the phenotype of the single mutant parent. This confirms that the new phenotype characterizes plants homozygous for both *flp* and *tmm*.

The double mutant had approximately the same frequency of clustering as did *tmm*, that is, 53% of all SUs had clustered stomata, but cluster size was slightly smaller. Compared with *tmm*, the double mutant had fewer clusters with 20 or more



**Figure 5.** Cryoscanning Electron Microscopy of the Wild-Type and Stomatal Mutants.

The abaxial epidermis of the cotyledons is shown.

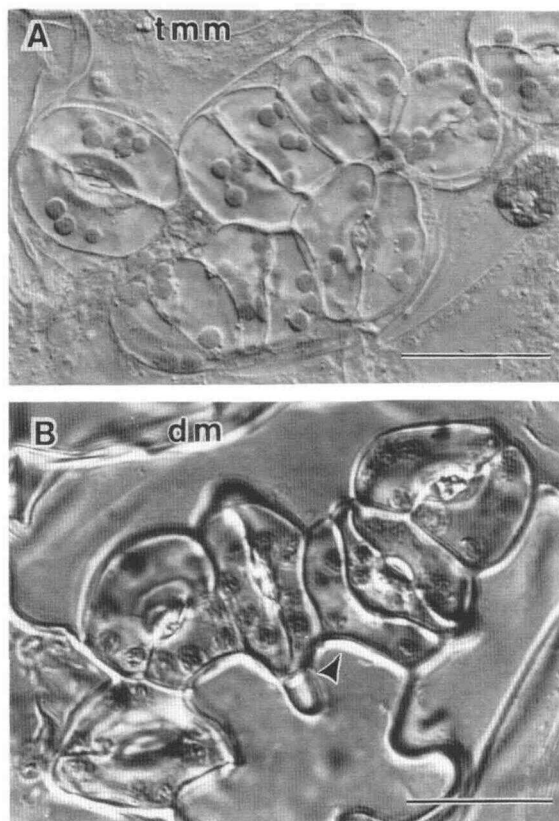
(A) Wild type (WT). All SUs consist of a single stoma, and no stomata are in contact with each other.

(B) *tmm*. SUs with many adjacent stomata have various arrangements, including arcs (top and bottom left). Pore development is arrested or incomplete in some smaller stomata.

(C) *flp*. The arrowheads indicate paired stomata. The pores are roughly parallel in paired stomata that are laterally arranged.

(D) Double mutant (dm). The arrowhead (upper right) indicates an SU with three adjacent guard cells. Stomata in clusters are either laterally aligned (arrow) or in arcs.

Bars = 50  $\mu\text{m}$  for (A) to (D).



**Figure 6.** Stomatal Clusters.

(A) shows an epidermal peel, and (B) shows a hand section of the abaxial epidermis of unfixed cotyledons viewed with differential interference contrast optics.

(A) *tmm*. The cluster consists of an even number (16) of guard cells. Pore development is incomplete in some stomata.

(B) Double mutant (dm). The arrowhead indicates an unpaired guard cell that is part of a cluster. Large clusters with an odd number of guard cells are a unique feature of the phenotype of the double mutant. Bars = 20  $\mu$ m for (A) and (B).

guard cells (Figure 7) and slightly fewer guard cells per SU or per cluster (Figures 9A and 9B). The double mutant also differed from *tmm* (and resembled *flp*) in having odd-numbered SUs (Figures 5D and 6B). Compared with *flp*, the double mutant had more and larger stomatal clusters, much larger clusters with an odd number of guard cells, and no single unpaired guard cells.

Cluster morphology in the double mutant had features of both parents. As in *tmm*, some clusters consisted of large groups of stomata. But unlike *tmm*, some clustered stomata were positioned laterally (Figures 4D and 5D), a feature also found in *flp*. Clusters in the double mutant tended to be more compact, with fewer spiral patterns than in *tmm* clusters.

Thus, the phenotype of the double mutant in the cotyledon is neither identical to either mutant parent (epistasis) nor sim-

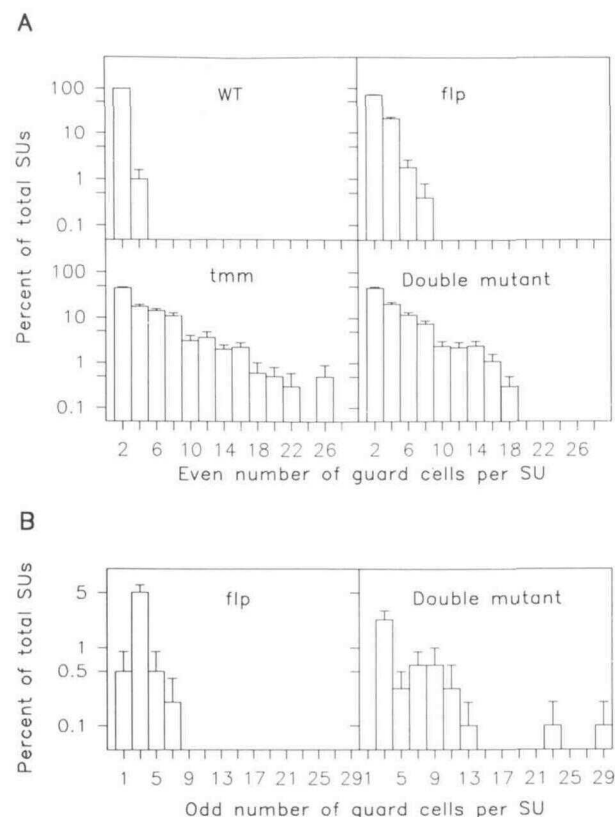
ply the sum of the phenotypes of the parents. Instead, some features of the double mutant resemble one or the other parent, and other features are somewhat intermediate between the two parents.

### Quantitative Analysis of Cotyledon Phenotypes

To assess how stomatal production was affected in cotyledons, the densities and absolute numbers of stomata, SUs, and epidermal cells were determined. The trends reported below appear representative of laboratory-grown plants (see Methods).

#### Stomata

All three mutant genotypes (*tmm*, *flp*, and the double mutant) have more stomata than does the wild type (Figure 10).

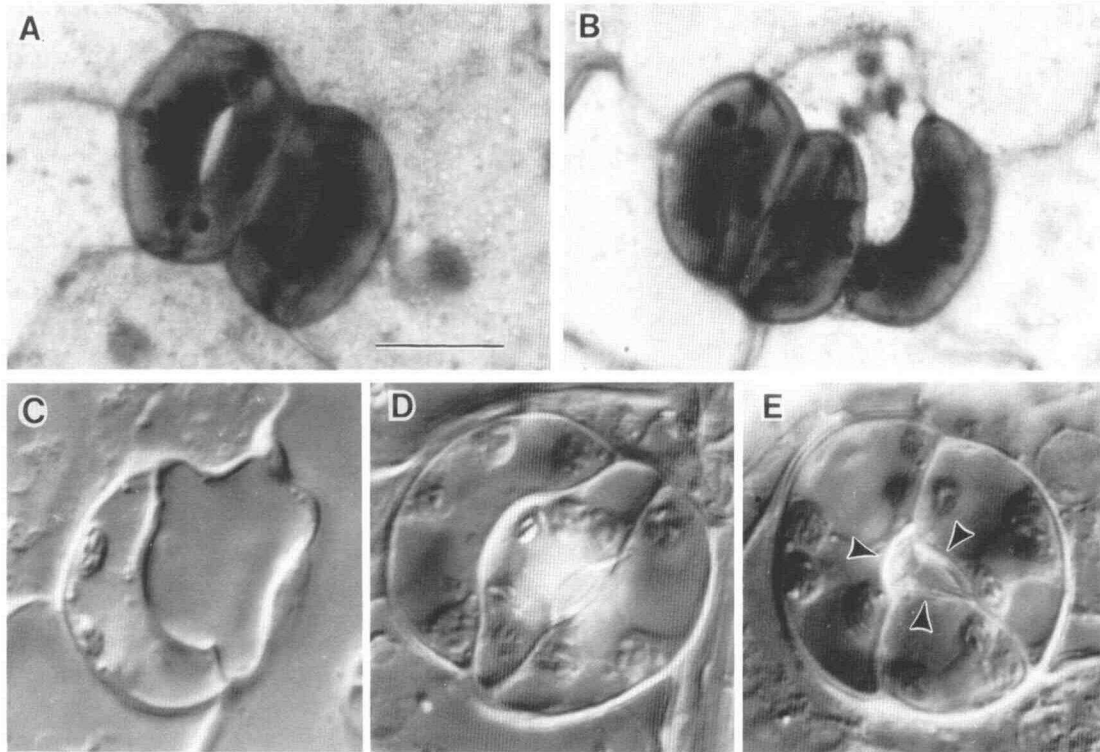


**Figure 7.** Frequencies of SUs with Even or Odd Numbers of Guard Cells.

Data from the abaxial epidermis of the cotyledon, with the logarithmic scale on ordinate. Standard error bars are shown.

(A) All four genotypes (wild type [WT], *flp*, *tmm*, and the double mutant) have SUs with an even number of guard cells.

(B) Only *flp* and the double mutant contain SUs with an odd number of guard cells.



**Figure 8.** Light Microscopy of Abnormal SUs.

Peels of the abaxial epidermis of cotyledons were stained with crystal violet, as shown in (A) and (B), or examined unstained using differential interference contrast optics, as shown in (C) to (E).

(A) to (D) *flp*. SUs contain four (A), five (B), one (C), and three (D) guard cells.

(E) Double mutant. A rare SU is shown that has one normal and two "half" (truncated) guard cells surrounding one stomatal pore (arrowheads). Bar in (A) = 10  $\mu$ m for (A) to (E).

**Table 1.** Genetic Analysis of *flp* and *tmm*

Cross	F <sub>1</sub>	F <sub>2</sub>	Segregation Ratios
	Phenotype	Phenotypes	
<i>flp</i> × WT <sup>a</sup>	WT (32) <sup>b</sup>	WT: <i>flp</i>	3.1:1 <sup>c</sup> (235:75)
<i>tmm</i> × WT	WT (6)	WT: <i>tmm</i>	2.9:1 <sup>c</sup> (211:73)
<i>flp</i> × <i>tmm</i>	WT (21)	WT: <i>flp</i> : <i>tmm</i> :dm <sup>d</sup>	8.5:3.3:3.5:1 <sup>e</sup> (384:147:157:45)

<sup>a</sup> Wild type.

<sup>b</sup> Number of plants.

<sup>c</sup>  $\chi^2$  test shows empirical ratio conforms to the expected ratio of 3:1 ( $P \geq 0.75$ ).

<sup>d</sup> Double mutant.

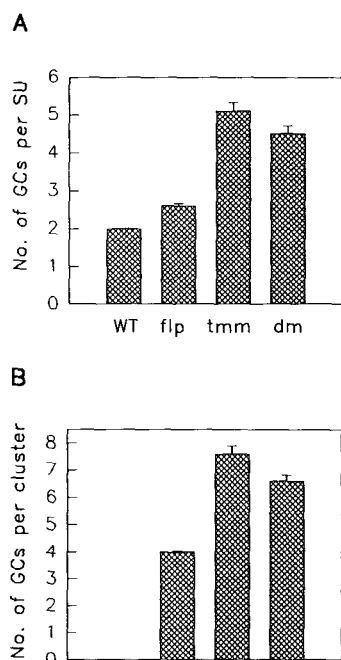
<sup>e</sup>  $\chi^2$  test shows empirical ratio conforms to the expected ratio of 9:3:3:1 ( $P > 0.10$ ).

### Stomatal Units

Both *tmm* and the double mutant have more SUs than does the wild type (absolute and density basis; Figure 10). Because each SU comes from at least one meristemoid, these data show that *tmm* and the double mutant produce more meristemoids in the cotyledon than does the wild type. Although not quantified, SU density also appeared higher in developing (as opposed to fully expanded) *tmm* cotyledons (Figure 3B). In *flp*, the absolute number of SUs was greater than that of the wild type in the experiment shown in Figure 10, but this result was not found consistently in all experiments.

### Epidermal Cells

Although cell size was not measured, there was no obvious difference in EC area among the four genotypes. Cotyledon area did not differ significantly among *tmm*, the double mutant,



**Figure 9.** Mean Number of Guard Cells per SU by Genotype.

(A) All SUs.

(B) Only clustered SUs included.

Values within (A) and (B) are different from one another ( $P < 0.05$ ). GCs, guard cells; WT, wild type; *dm*, double mutant.

and the wild type (see legend of Figure 10). Both the absolute number and the density of ECs decreased in the double mutant compared with the wild type (Figure 10). The absolute number, but not the density, of epidermal cells in *flp* was greater than that in the wild type. When the data were expressed as a ratio, that is, as the number of ECs per SU (Figure 11), only the means of *tmm* and the double mutant were different from those of the wild type; both produced fewer ECs relative to SUs. The double mutant produced even fewer ECs per SU than did *tmm*. Thus, the *tmm* mutation upregulates the production of meristemoids and stomata, and downregulates the production of epidermal cells in cotyledons.

#### Mutant Appearance and Stomatal Phenotypes in Other Organs

*flp*, *tmm*, and *tmm flp* plants all looked relatively normal and healthy under the growing conditions tested. Growth conditions included continuous light from fluorescent lamps in sterile or pot culture, or essentially seasonal daylight and photoperiod in greenhouse pots.

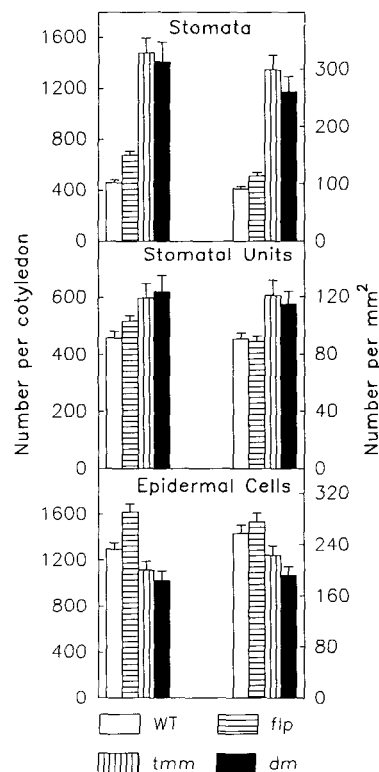
Various organs were examined using hand sections, epidermal peels, whole mounts, and cryoscanning electron microscopy to determine semi-quantitatively the extent of

stomatal clustering throughout the plant. In *tmm* and the double mutant, the clusters appeared to be larger and more numerous in the abaxial epidermis of the cotyledon than in most leaves. In *flp*, the frequency of occurrence of abnormal SUs, mainly paired stomata, seemed comparable between the cotyledon and all leaves.

In wild-type and *flp* plants, stomata were distributed throughout the inflorescence stem (Figure 12A). However, in *tmm* and the double mutant, stomata were absent or rare on the inflorescence stem (Figure 12B). Thus, in *tmm* and the double mutant, there was a proliferation of stomata and of SUs (meristemoids) in the cotyledon and an almost complete suppression of stomatal formation in the inflorescence stem.

#### DISCUSSION

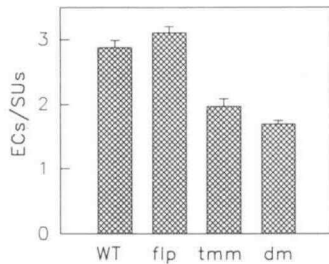
*tmm* and *flp* are novel *Arabidopsis* mutations that affect stomatal development. Each mutation is recessive, and each has clustered (extra adjacent) stomata in the cotyledons. Both mu-



**Figure 10.** Absolute Numbers and Densities of Stomata, SUs, and ECs.

Data from the abaxial epidermis of cotyledons. Bars indicate standard errors. In this sample, cotyledon areas were equivalent in the wild type (WT), *tmm*, and the double mutant (*dm*) (with a range of 5.1 to 5.3 mm<sup>2</sup>); *flp* cotyledons were larger (6.0 mm<sup>2</sup>,  $P < 0.05$ ).





**Figure 11.** Mean Number of ECs per SU.

The means between *tmm*, the double mutant (*dm*), and the wild type (WT) are different ( $P < 0.05$ ); the means of *flp* and the wild type are the same. Thus, *tmm* and the double mutant produce fewer ECs relative to SUs than do the wild type or *flp*.

tations influence stomatal production, possibly by altering precursor cell (meristemoid) activity. The *TMM* gene product also controls stomatal production by regulating meristemoid formation. Thus, both mutations alter the wild-type pathway of stomatal development, and *TMM* operates at several developmental levels.

### Stomatal Development in Arabidopsis

Arabidopsis seems to follow the pattern of stomatal formation deduced for other members of the Brassicaceae (Figure 1; Paliwal, 1967; Pant and Kidwai, 1967; Landré, 1972). Certainly, stomatal meristemoids are present in and divide in Arabidopsis (Figures 2 and 3; Wei et al., 1994). However, the reconstruction of cell lineage solely from the examination of cell wall

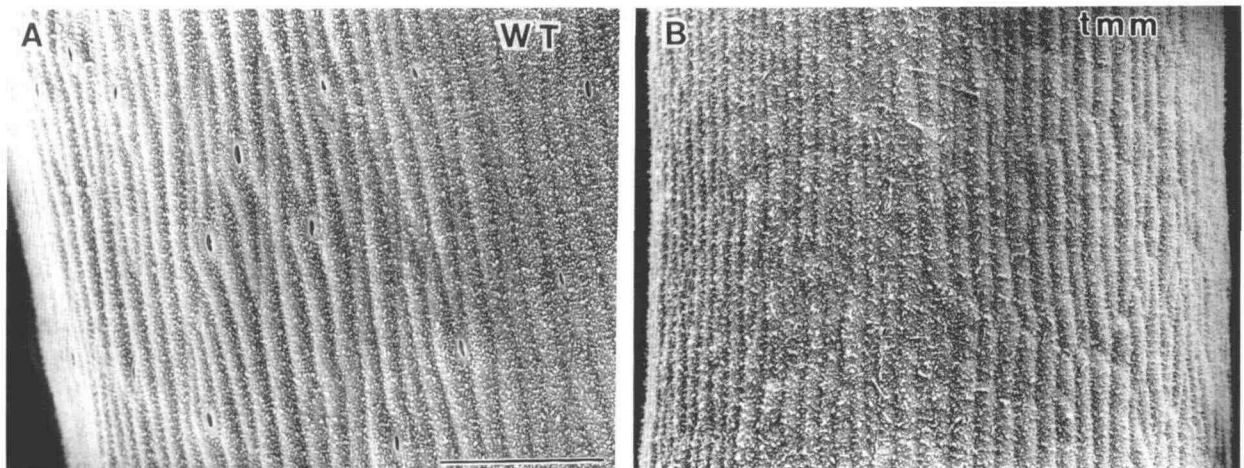
patterns is no substitute for study of the same cells through time, for example, through the use of dental impression material. Indeed, such studies with other genera have shown that there is some variability in the formation and activity of meristemoids (Sachs, 1991; Sachs and Novoplansky, 1993). Thus, a full assessment of wild-type development will require more intensive study of cell lineages.

Nevertheless, it is likely that wild-type stomatal development in Arabidopsis cotyledons and leaves can be divided into at least five stages (Figures 1 and 2): (1) the patterning and initiation of meristemoids; (2) one to several unequal divisions of the meristemoids to form nonstomatal ECs; (3) the conversion of the meristemoids to guard mother cells; (4) the symmetrical division of the guard mother cells into two young guard cells each; and (5) the differentiation of guard cells to form stomata. Our data indicate that *TMM* and *FLP* might act during several of these stages to influence stomatal production.

### *flp* and *tmm* Primarily Affect Stomatal Production

These mutations in both the *TMM* and *FLP* genes resulted in clustered stomata. Clusters in *tmm* contain an even number of guard cells, whereas *flp* has many paired stomata as well as some clusters with an odd number of guard cells. The presence of clusters shows that a primary effect of both mutations is on stomatal production; both *flp* and *tmm* have many more stomata than does the wild type (Figure 10). Based on these data, it appears that the effects of these mutations on stomatal spacing and differentiation may be secondary.

Stomatal differentiation is sometimes abnormal in both mutants, including arrested pore development and distorted guard cell shape. These abnormalities are frequently found in *tmm*



**Figure 12.** Cryoscanning Electron Microscopy of the Inflorescence Stem.

(A) Wild type (WT) stems have stomata.

(B) *tmm* stems have no stomata.

Bar in (A) = 100  $\mu$ m for (A) and (B).

in smaller stomata tightly packed in clusters and apparently produced after the surrounding cells have expanded. However, most of the stomata in clusters differentiate normally, and even unpaired guard cells in *flp* usually look essentially like wild-type guard cells. Thus, it is possible that instances of abnormal differentiation are a consequence of stomatal overproduction and crowding. If so, neither *TMM* nor *FLP* plays a direct role in guard cell differentiation. Detailed study of stomatal structure and gene expression will be required to confirm this hypothesis.

Stomatal spacing is dramatically altered in both mutants. The presence of adjacent stomata in these mutants violates the most orderly aspect of wild-type stomatal spacing (Sachs, 1991), that is, there is no stoma-free region around many mutant stomata. However, the presence of stoma-free regions around stomatal clusters raises the possibility that neither mutation affects primarily the patterning of meristemoids. This interpretation is supported by the (nonquantitative) observation that the stomatal clusters seem to be distributed in a pattern that resembles the wild type, with each cluster or single guard cell positionally equivalent to a single stoma in the wild type. Thus, the presence of adjacent stomata within a cluster might result from an overproduction of stomata from each meristemoid rather than from altered patterning of the meristemoids themselves. If so, neither *TMM* nor *FLP* plays a direct role in stomatal patterning, and they only secondarily influence stomatal spacing, making it likely that both mutations affect primarily the production of stomata.

#### ***flp* May Act in Later Stages of the Meristemoid Pathway**

After stomatal meristemoids produce one or more ECs, the meristemoid normally converts into a guard mother cell. The *flp* mutation may alter later stages of this pathway. The ratio of ECs to SUs in *flp* cotyledons is not different statistically from the wild type (Figure 11), indicating that *flp* meristemoids produce a full complement of ECs. Similarly, *flp* SU density is comparable with that of the wild type (Figure 10). Thus, based on these data, neither precursor cell (meristemoid) formation nor early meristemoid activity appears altered in *flp*.

It is likely that all stomata in a *flp* cluster share the same cell lineage, that is, that they are produced from the same meristemoid. This hypothesis is supported by the small size of *flp* clusters (typically two to four stomata) and by the observation that paired stomata are often laterally aligned and at roughly the same stage of development.

These data also argue against *flp* clusters forming from a breakdown in lateral inhibition. The classical lateral inhibition hypothesis for stomatal spacing holds that a wild-type meristemoid and/or guard mother cell produces some sort of field that prevents previously formed, nearby cells from developing into stomata (Bünning, 1965).

A viable hypothesis is that *flp* acts by altering cell cycling after the meristemoid becomes committed to forming guard

cells and after it has produced a full complement of ECs. This could result in the production of, for example, one to four guard cells, depending on how many times the committed cell has divided. In any case, it is likely that the *FLP* locus controls the coordinated production of guard cells since the *flp* mutation results in the formation of unpaired guard cells.

Clearly, analysis of the action of both *flp* and *tmm* requires the isolation of more alleles, because the severity of existing alleles is unknown. The presence of many wild-type-like SUs (normal, unclustered stomata) in both *flp* and *tmm* could suggest that the alleles described here are hypomorphic ("leaky").

#### ***tmm* May Alter the Fate of Cells Produced by Meristemoids**

The *tmm* mutation results in the production of fewer ECs relative to SUs (Figure 11). Thus, if each *tmm* cluster derives from one meristemoid, then the extra guard mother cells might be produced at the expense of ECs. In the wild type, the meristemoid converts to a guard mother cell after a full complement of ECs is produced. In *tmm*, this conversion might occur earlier, producing extra guard mother cells. Thus, guard mother cells would be substituted precociously for ECs, and *tmm* would influence the fate of the cells produced by the meristemoid.

Clusters might also form by other means in *tmm*. For example: (1) extra guard mother cells might also be produced for a more extended period than they are in the wild type; (2) clusters might result from a breakdown in lateral inhibition; and (3) clusters could form from several meristemoids if the formation of intervening ECs were repressed.

Distinguishing among these possibilities will probably require study of cell lineages in the same region of the cotyledon through time (Sachs and Novoplansky, 1993). Present data are consistent with the possibility that *tmm* alters meristemoid activity at least in part by producing extra guard mother cells at the expense of ECs.

#### ***tmm* Also Affects Entry into the Pathway**

Several lines of evidence show that the *tmm* mutation affects the production of meristemoids by influencing entry into the pathway of stomatal development. Cotyledons of the *tmm* mutant consistently have more SUs than do cotyledons of the wild type (Figure 10). Because it is likely that each SU derives from at least one meristemoid, *tmm* appears to upregulate precursor cell formation in the cotyledon. In the *tmm* inflorescence stem, stomata (and apparently meristemoids) are virtually absent, whereas in the wild type, stomata are distributed throughout the stem (Figure 12). Thus, in the stem, the *tmm* mutation prevents entry into the pathway leading to stomatal formation. In addition, in *tmm*, some reproductive organs have domains in which the number of SUs is augmented, and others have domains where stomata and meristemoids are completely absent (M.J. Geisler and F.D. Sack, unpublished data).

Because *tmm* is recessive and likely to be a loss-of-function mutation, it is likely that the *TMM* gene regulates the formation of meristemoids and that the direction of regulation depends on the organ. The *TMM* gene product appears to limit the number of meristemoids produced in the cotyledon and to promote the formation of meristemoids in the stem.

### ***TMM* Is Critical for Stomatal Formation**

The *TMM* gene regulates stomatal production at several developmental levels. It regulates entry into the pathway, that is, meristemoid formation; it also restricts meristemoid activity so that only one guard mother cell is normally produced (and the formation of clusters is prevented). The *TMM* gene product therefore appears to play several critical roles in the pathway that results in stomatal production.

Stomatal development has some intriguing parallels with bristle development in *Drosophila*, such as the selection of an EC as a sensory organ precursor cell that divides and gives rise to different cell types, especially neurons (Campos-Ortega, 1993). Complex interactions between inhibitors and promoters determine the selection of the sensory organ precursor cell as well as the fate of the cells it produces. Mutations in important neurogenic genes, such as *Notch* and *Delta*, regulate (increase) neuron number by both forming more precursor cells and converting more progeny cells to neurons (Posakony, 1994).

The *tmm* mutation is similar to these mutant bristle phenotypes in that *tmm* alters the number of precursor cells as well as the numbers of key types of cells produced from the precursor cell. This parallel is consistent with the possibility that *TMM* is an important regulator of stomatal formation.

### **Double Mutant**

The relationships between the *FLP* and *TMM* gene products appear complex. In the cotyledon, neither mutation is epistatic; the double mutant has features of both parents, but this combination is not obviously additive. Instead, several features are roughly intermediate in character, indicating that these loci interact. However, in the stem, *tmm* is epistatic to *flp* in that both *tmm* and the double mutant lack stem stomata. These relationships will probably be clarified through analysis of the phenotypes of other alleles.

### **Stem Stomata Appear Unnecessary**

A detailed quantitative study is in progress to determine the distribution of both the *flp* and *tmm* phenotypes throughout the plant. Initial observations show that at least some extra adjacent stomata are present in all leaves of both genotypes. It remains to be determined whether stomata in clusters are functional and whether clustering affects gas exchange.

Nevertheless, it is already clear that an absence of stomata in the inflorescence stem in *tmm* and the double mutant still allows vigorous seed set, at least in laboratory- or greenhouse-grown, relatively unstressed plants. Thus, inflorescence stem stomata are not necessary for reasonable reproductive productivity in Arabidopsis.

### **Other Abnormalities and Mutants**

Stomatal abnormalities reminiscent of both mutants occur, albeit rarely, in wild-type plants of various genera (Dehnel, 1961; Pant and Kidwai, 1967; Inamdar et al., 1969) and could result from malfunctioning of homologs of *TMM* and *FLP*. Some trichome phenotypes in Arabidopsis, as in *tmm* and *flp*, show extra adjacent cells. Clustered trichomes occur both in the *Tryp-tichon* mutant and when a trichome determination gene (*GLABROUS1*) is overexpressed in plants heterozygous for a strong *transparent testa glabra* allele (Hülkamp et al., 1994; Larkin et al., 1994). The developmental mechanisms producing clustering in these systems are not known.

We are aware of only one other published report of a stomatal mutant, the *eceriferum-g* mutant of barley, which has extra adjacent stomatal complexes (Zeiger and Stebbins, 1972). This mutant also has an altered cuticle, which suggests that at least in grasses, a gene affecting stomatal formation also acts in other developmental pathways. Although possible pleiotropic effects in *tmm* and *flp*, such as effects on substomatal cavity formation, are still being investigated, the only clear effect found to date in these mutants involves stomatal development.

### **Conclusions**

Mutations have been identified at two different loci, *FLP* and *TMM*, with the phenotype of extra adjacent stomata. The genes appear to act in different ways, yet both appear to be required for restricting the production of guard mother cells to one from each precursor cell. The *TMM* gene product also regulates entry into the pathway by controlling precursor cell formation. Thus, *TMM* regulates stomatal production at several developmental levels.

### **METHODS**

#### **Plant Material and Culture**

M<sub>2</sub> seeds from ethyl methanesulfonate-mutagenized seeds of *Arabidopsis thaliana* Columbia ecotype (Lehle Seeds, Round Rock, TX) were used for screening. The line lacked trichomes because it was homozygous for *glabrous1* (Marks and Feldmann, 1989). M<sub>1</sub> seeds were produced by imbibition in 0.13% ethyl methanesulfonate for 10 hr at 23°C. For screening, M<sub>2</sub> seeds were surface sterilized and sown on 1% agar containing the nutrients described by Haughn and Somerville (1986) plus 2% sucrose. Gridded square Petri plates containing the seeds were sealed with porous surgical tape. Seedlings were grown

under continuous 50 to 100  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  photosynthetically active radiation provided by cool-white fluorescent tubes and maintained at  $\sim 22^\circ\text{C}$ . To allow seed set, some putative mutant seedlings were transferred to Magenta GA7 boxes (Sigma) with the same medium and growing conditions. For crosses and mutant characterization, plants were grown in pots in a soil mix that included peat, perlite, and vermiculite.

### Mutant Screening

For screening, cotyledons were examined when the seedlings were 12 to 20 days old. Cotyledons were collected using sterile technique. The abaxial epidermis was examined either in unstained and fresh whole mounts of the cotyledon or in epidermal "peels." Peels were obtained by pressing the cotyledon onto double-stick tape affixed to a microscope slide and then scraping off the bulk of the cotyledon using a razor blade. The resulting abaxial epidermis was then stained with 0.05% toluidine blue in 0.05% borate or with 0.05% crystal violet. The stomatal phenotype was evaluated at  $\times 400$  magnification using bright-field microscopy. Over 21,000  $M_2$  seedlings from  $\sim 7000$  parental ( $M_1$ ) plants were screened.

### Quantitative Characterization of Phenotypes

The data reported (Figures 7 and 9 to 11) are from one representative experiment of four experiments. For each experiment, one cotyledon was collected from each of at least 20 plants for each genotype. Fresh whole mounts of intact cotyledons were examined.

The total numbers of stomata, stomatal units (SUs), and epidermal cells (ECs) were determined in each of seven randomly selected fields per cotyledon. Each field represented 0.03  $\text{mm}^2$  of the cotyledon surface. Totals of the seven fields were used to calculate densities.

For scoring purposes, an SU was defined as all those stomata actually in contact with one another. Thus, some SUs consisted of linear or helical groups and some of tight clusters. Each single stoma or single isolated guard cell was also scored as an SU, regardless of genotype. All (nonsomatal) ECs were scored together, regardless of whether they were or were not in contact with guard cells.

Cotyledon area was determined using tracings (magnification  $\times 60$ ) analyzed with an area meter (model LI-3100; Li-Corp., Inc., Lincoln, NE). Cotyledon area was used to calculate the total (absolute) numbers per cotyledon of stomata, SUs, and ECs, and frequencies of SUs with varying numbers of guard cells, from the previously derived density data.

All quantitative differences reported were statistically significant at a 0.05 level ( $t$  test).

### Microscopy

Light micrographs of epidermal peels were taken using bright-field or differential interference contrast optics with an IM 35 microscope (Carl Zeiss, Inc., Thornwood, NY) and photographed using Kodak Technical Pan film. Whole mounts of cotyledons cleared in 95% ethanol for at least 24 hr were also examined.

All scanning electron micrographs were of frozen material using a Cambridge Scanning Electron Microscope (MK IV, Cambridge, UK) equipped with a Hexland CT1000 Cryotransfer apparatus (Oxford Instruments, Oxford, UK). Tissue was frozen in liquid nitrogen, sublimed at  $-85^\circ\text{C}$ , coated with gold, and then examined at approximately  $-180^\circ\text{C}$ .

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