

Divergent regulation of stomatal initiation and patterning in organ and suborgan regions of the *Arabidopsis* mutants *too many mouths* and *four lips*

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Abstract. Stomata are consistently patterned so that they are not in contact. This patterning is violated in the *too many mouths* (*tmm*) and *four lips* (*flp*) mutations of *Arabidopsis thaliana* (L.) Heynh. which have stomatal clusters in the first-formed leaves. To clarify the function of both genes in stomatal initiation and patterning, the phenotypes of many different organs were quantified. The *flp* mutation affects dorsiventral and cylindrical organs differentially with respect to the frequency of clustering. The *tmm* mutation has a more complex region-specific phenotype in that some regions lack stomata entirely, other regions have excess stomata, and the flower stalk exhibits an apex-to-base gradient from excess to no stomata. This suggests that *TMM* represents an unusual type of gene regulating plant cell development in that it can either influence stomatal initiation in a positive or negative fashion depending on region. Since the frequencies of initiation and clustering can be uncoupled in *tmm*, these two functions are under separate region-specific control. Analysis of double mutants shows that *tmm* and *flp* in some cases show region-specific interactions in both cluster formation and initiation, and that there may be subpopulations of stomata under different genetic control.

Key words: *Arabidopsis* (mutants) – Cell differentiation – Epidermis – Mutant (*Arabidopsis*, stoma) – Stoma (development, patterning)

Introduction

Stomata are found in the epidermis of most aerial plant organs and are necessary for plant life on land. They

regulate gas exchange for photosynthesis by opening or closing a pore between two guard cells. Like trichomes – another type of specialized epidermal cell – stomata are not randomly distributed but are patterned in that they are almost never adjacent to each other in mature organs (Bünning 1953; Larkin et al. 1997). Identification of the genes and mechanisms involved in stomatal development and patterning is important for understanding both plant cell differentiation and one of the bases of plant productivity.

Stomatal precursor cells, termed meristemoids in dicotyledonous plants, are produced in an asymmetric division and may continue to divide asymmetrically (Fig. 1; Bünning 1953; Larkin et al. 1997). Each meristemoid eventually converts into a guard mother cell which divides symmetrically to form the two guard cells that make up one stoma. The oriented production of epidermal cells by the meristemoid can generate a stomatal spacing pattern and appears to involve cell-cell signaling. For example, meristemoids that form adjacent to each other subsequently divide to produce intervening epidermal cells (Sachs et al. 1993; Larkin et al. 1997). Patterning has also been hypothesized to result from lateral inhibition originating from developing or mature stomata (Bünning 1953; Korn 1993; Boetsch et al. 1995). However, relatively little is known about the genes and mechanisms involved in stomatal patterning.

Several mutations are known to affect stomatal patterning in *Arabidopsis*, and two of these, *too many mouths* (*tmm*) and *four lips* (*flp*), were identified by the presence of stomata in contact in the cotyledon (Yang and Sack 1995; Larkin et al. 1997). In cotyledons, *tmm* has large clusters of stomata, while *flp* has smaller clusters with some unpaired guard cells. The *tmm* mutation promotes stomatal initiation (meristemoid formation) in cotyledons since these organs have many more stomata (after adjustment for clustering) than the wild type (WT). In contrast, *tmm* suppresses initiation in stems since stomata are mostly absent in stems of the inflorescence (Yang and Sack 1995).

Tissue- and region-specific control of initiation has also been reported for trichomes. Both the *gll* and *ttg*

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Abbreviations: SU = stomatal unit; WT = wild type

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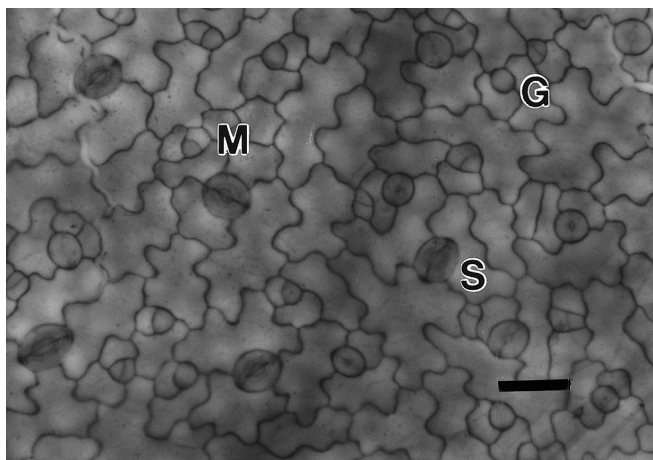


Fig. 1. Pattern of stomata and asynchronous stomatal development in an expanding first leaf of *Arabidopsis* that was cleared and stained. Note simultaneous presence of meristemoids (*M*), more rounded guard mother cells (*G*) and mature stomata (*S*). Some meristemoids form in cells that neighbor stomata. Bar = 20 μm

mutants of *Arabidopsis* eliminate trichome formation on leaf blades, but do not affect the presence of trichomes on the leaf margin (Hülkamp et al. 1994; Larkin et al. 1997). In addition to suppressing trichome formation, *ttg* also upregulates root hair initiation (Galway et al. 1994). In contrast, the *tmm* mutation affects only stomatal formation, but shows both upregulation (in cotyledons) and suppression (in inflorescence stems; Yang and Sack 1995). Unlike *ttg* or *gll*, which affect all trichomes in the leaf blade, *tmm* causes the clustering of only some of the stomata, and other stomata are patterned normally in cotyledons. However, a more complete evaluation of the functions of TMM and FLP requires knowledge of mutant phenotypes throughout the plant, not just for cotyledons and inflorescence stems.

The objectives of this study were to evaluate most organs of the plant by quantifying the extent of stomatal initiation and clustering in *tmm* and *flp* mutants, and to identify other instances of regional-specific regulation using both existing and new alleles at each locus. The *flp/tmm* double mutant was analyzed to determine the extent and nature of gene interaction and to shed light on whether all stomata are affected equally by each mutation. Here we report that the stomatal initiation phenotype of *tmm* varies in many parts of the plant in a locally predictable and specific manner, and that the effect of *flp* on cluster formation depends upon organ shape. Analysis of the double mutant reveals additional functions of *flp* and identifies regions and developmental pathways where *flp* and *tmm* do and do not appear to interact.

Materials and Methods

Plant material and culture. New alleles were obtained by EMS mutagenesis (*flp-2*, *flp-3*, and *flp-4*) and by fast-neutron bombardment (*tmm-2*). Except for the *flp-2* allele which is in a C24

background, all alleles are in the Columbia ecotype of *Arabidopsis thaliana* (L.) Heynh. Since all alleles showed the same region-specific patterns as *flp-1* and *tmm-1* (the alleles originally described in Yang and Sack 1995), only the latter were used for quantitative analysis. Double mutants of *tmm-1/flp-1* were verified by backcrossing separately to *tmm-1* and *flp-1* as described in Yang and Sack (1995). All plants were grown under continuous “Coolwhite” fluorescent lamps (Grey Supply Co., East Chicago, Ind., USA) with a light flux of $130 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at soil level, on Promix (Hummert International, Earth City, Mo., USA) potting soil.

Histology. Fully expanded organs were cleared in 85% lactic acid by autoclaving for 15 min and then leaving at room temperature overnight. The organs were then rinsed, stained with haematoxylin, dehydrated, and mounted in Permount (O’Brien and McCully 1981). The area of 10 cotyledons was measured before and after dehydration, and the shrinkage was the same for all genotypes, a 20% reduction in area. Chemicals were obtained either from Sigma (St. Louis, Mo., USA), or Fischer (Fair Lawn, N.J., USA).

Sampling and scoring. To quantify stomatal production, “stomatal units” (SUs) were operationally defined as all guard cells in contact. Thus, a cluster or a normal isolated stoma was each scored as a stomatal unit. Sampling was based on microscope fields of 0.332 mm^2 (20–50 stomata per field depending on genotype and organ). To determine the accuracy of using field sampling to estimate the total number of SUs, data derived using fields were compared to direct measurement of all of the SUs in the entire cotyledon for the same samples of five cotyledons for each genotype. There were no significant differences in the values for the total number of SUs derived from these two methods.

For organs other than the cotyledon, either the entire epidermis was scored, or five fields were chosen randomly. For the inflorescence stem, no significant differences were detected between fields sampled from subapical, or basal regions, or from branches. Because *tmm* and double-mutant flower stalks and siliques exhibited gradients in stomatal distribution, each organ was sub-divided into 0.3-mm segments and all stomata were scored in each segment. Because WT and *flp* flower stalks and siliques had an even distribution of stomata, stomata were scored in representative fields.

Total SU number was determined to be a more reliable estimate of stomatal initiation than organ area. The surface area of fully expanded organs varied considerably within a genotype, even in synchronously grown populations. In contrast, the values for total SU number showed much lower variability within genotypes (Table 1; data not shown), and organ area did not correlate with total SU number (data not shown). Also, organ areas were not significantly different between genotypes (*tmm* and double-mutant siliques and flower stalks were an exception) whereas total SU number revealed reliable differences. Since each SU derives from at least one meristemoid, the total number of SUs is a minimal index of stomatal initiation.

Two parameters were used to measure the extent of stomatal clustering in different organs. Cluster frequency (F) was calculated as the number of “abnormal” stomatal units (with other than two guard cells) in each field divided by the number of all SUs in that field. Cluster size (S) was calculated as the mean number of guard cells in all abnormal SUs per field. All differences reported are statistically significant (Student *t*-test, $P < 0.05$).

Results

Organ- and region-specific effects on precursor cell formation

The flp mutant mostly resembles the WT in stomatal initiation. Although most *flp* organs have clustered stomata, with respect to the total number of SUs in

Table 1. Mean total number of stomatal units (\pm SE, $n = 15\text{--}20$ for each organ)

Organ	WT	<i>flp</i>	<i>tmm</i>	<i>tmm/flp</i>
Anther	20 \pm 1	16 \pm 2 ^c	22 \pm 1 ^c	24 \pm 1 ^d
Sepal, Ab.	197 \pm 15	200 \pm 10	386 \pm 50 ^d	411 \pm 30 ^d
Sepal, Ad.	85 \pm 4	65 \pm 10	0.3 \pm 0.3 ^d	0 ^d
Silique	927 \pm 70	913 \pm 60	130 \pm 20 ^d	584 \pm 60 ^d
Fl. stalk	232 \pm 10	166 \pm 27 ^c	100 \pm 7 ^d	123 \pm 4 ^d
Caul., Ab.	17k \pm 6k ^a	21k \pm 2 k	11k \pm 2k ^d	11k \pm 2k ^d
Caul., Ad.	16k \pm 2 k	24k \pm 1 k	8k \pm 2k ^d	7k \pm 2k ^d
Cotyl., Ab.	481 \pm 8	408 \pm 8 ^c	746 \pm 20 ^d	705 \pm 20 ^d
Cotyl., Ad.	99 \pm 3	116 \pm 4	129 \pm 4 ^c	70 \pm 5 ^d
Infl. Stem ^b	24 \pm 2	21 \pm 2 ^c	0 ^d	0 ^d

^ak = thousands^bStomatal density is reported for the stem only (the number of SUs per 0.332-mm² field)^cSignificantly different from the WT ($P < 0.05$)^dSignificantly different from the WT ($P < 0.01$)

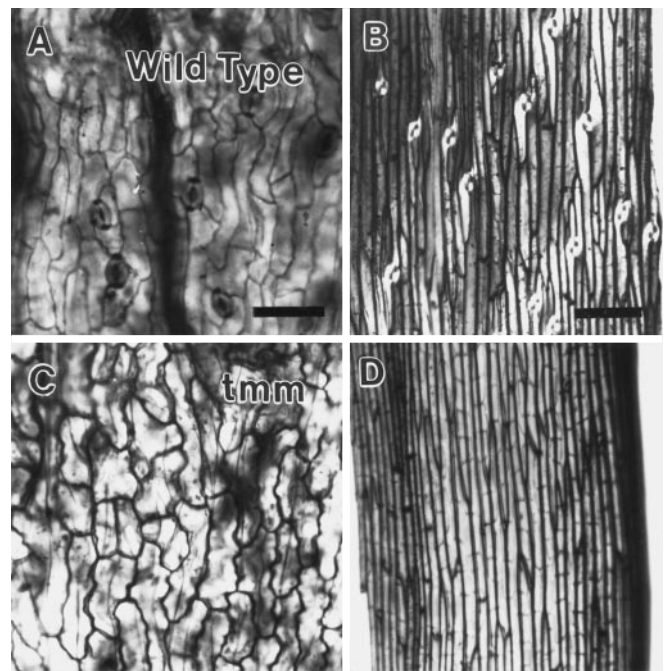
Ab. = abaxial, Ad. = adaxial, Caul. = cauline leaf, Cotyl. = cotyledon, Fl. stalk = flower stalk, Infl. stem = inflorescence stem

each organ, the *flp* phenotype is essentially comparable to the WT. Thus *flp* has stomata wherever the WT does, and the total numbers of stomatal units in *flp* siliques, cotyledons, and sepals are the same as for the WT (data not shown). Exceptions were the *flp* anther, inflorescence stem, and flower stalk, where SU number was slightly, but significantly lower than in the WT (Table 1). This suggests that in many organs *flp* plays no detectable role in meristemoid formation, while in others it slightly suppresses formation.

The *tmm* mutation affects the distribution and number of stomatal units. In contrast to *flp*, the *tmm* mutation strongly affects the extent of stomatal formation. The *tmm* mutation almost completely eliminates stomatal formation on the adaxial side of the sepal, in the inflorescence stem (Figs. 2, 3) and in the hypocotyl (data not shown). The number of SUs in cauline leaves, flower stalks, and siliques is also reduced by *tmm*. There was no significant difference in organ size between *tmm* and the WT except for siliques and flower stalks which were, on average, 50% smaller (data not shown). The smaller size of *tmm* flower stalks and siliques only partially contributes towards a reduction in SU number since average SU density was also decreased and even areas of *tmm* siliques with the highest SU density had lower values than the WT (data not shown).

In other regions, *tmm* increases the number of SUs compared to the WT (Table 1). This occurs in cotyledons (both abaxial and adaxial sides), anthers, and on the abaxial side of the sepal (Figs. 3, 4). Differential regulation of initiation is not simply organ specific, as SU number is enhanced on one side of the sepal (abaxial), and virtually eliminated on the other side (adaxial). However, in some organs, such as in *tmm* cotyledons, both sides show an increase in SU number and thus both sides are affected in the same way.

A striking aspect of the phenotype in *tmm* is the presence of a gradient in stomatal formation in the flower stalk. Complete suppression is found in the basal (stem) end, and enhanced stomatal initiation and clus-

**Fig. 2A–D.** Elimination of stomata in *tmm* (C, D) compared to the WT (A, B) on the adaxial side of sepals (A, C) and in inflorescence stems (B, D). Cleared and stained tissue. Bars = 20 μ m (A), 100 μ m (B)

tering are present in the apical (floral) end (Figs. 3, 5). In siliques there is a complete suppression of SUs at both ends, and a gradient of stomata with a peak density in the middle. In WT siliques and flower stalks, stomata are distributed evenly throughout the length. A second allele, *tmm-2* shows all the above patterns of regional specificity in stomatal distribution. Thus *tmm* strongly affects stomatal initiation and distribution. The direction and extent of this effect depends upon the organ, and in some organs opposite effects occur depending on the region.

Stomatal Unit Distribution

Wild type

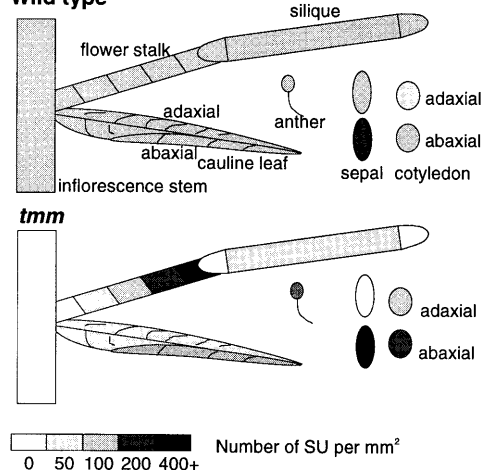


Fig. 3. Schematic representation of SU densities of WT and *tmm* inflorescences. Densities in the cotyledon are shown for comparison. Densities are grouped into segments along the lengths of the flower stalk and silique for scoring purposes; thus the density change is depicted as changing in steps rather than as a continuous gradient as in the actual plants

Stomatal cluster frequency and size

The clustering and initiation phenotypes are separable in different *tmm* organs. Clusters arise when extra stomata form adjacent to each other. However, many SUs are unaffected in *tmm*, i.e. they consist of a single stoma. The frequency with which *tmm* SUs are clustered (F) varies

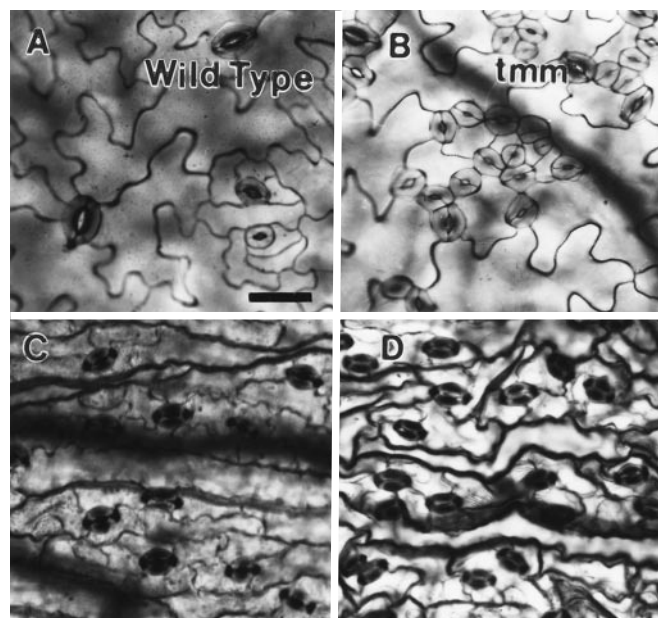


Fig. 4A–D. Enhancement of SU number in *tmm* (B, D) compared to the WT (A, C) on the abaxial side of sepals (C, D) and the abaxial side of cotyledons (A, B). The increase in SUs in *tmm* sepals (D) is accompanied by very little clustering. In *tmm* cotyledons there is an increase in clustering as well as in SU number (B) compared to the WT (A). Bar = 20 μ m

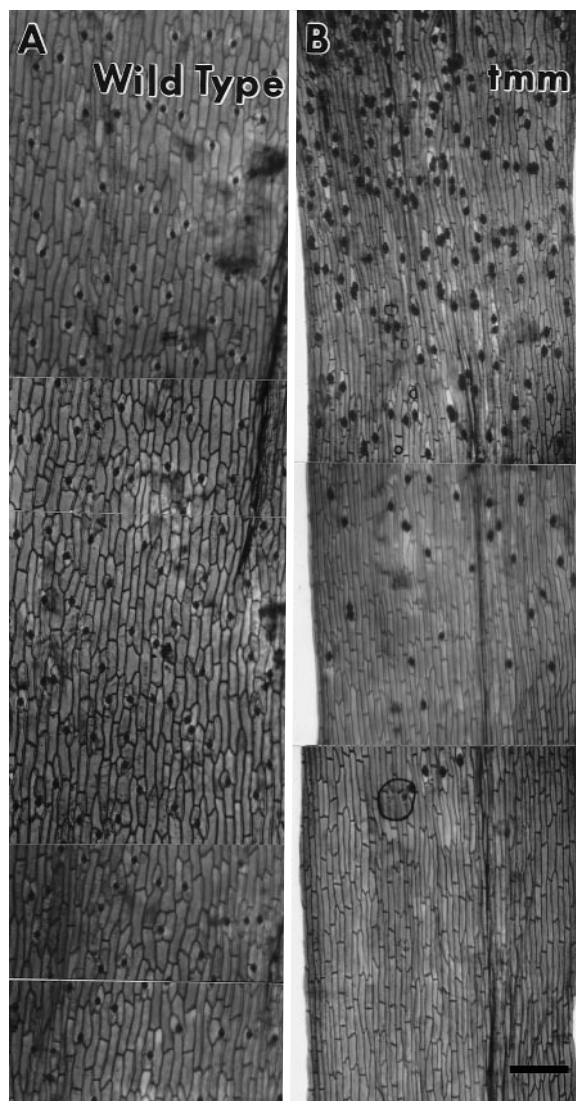


Fig. 5A–B. Entire flower stalks of the WT (A) and *tmm* mutant (B). The apical (floral) end of the *tmm* stalk (top) has more stomata than the WT, whereas the *tmm* base lacks stomata. The tissue in between shows an apex-to-base gradient from enhancement to suppression of SU formation. Note also the presence of some clusters at the apical end of *tmm*. Bar = 150 μ m

considerably depending on the type of organ (Table 2). Increases in the initiation of SUs do not always correlate with frequent clustering. For example, the abaxial epidermis of the *tmm* sepal has more than twice the total number of SUs as the WT, but very few (7%) of these SUs are clustered. In contrast, the abaxial epidermis of the *tmm* cotyledon has 50% more SUs than the WT, and over 65% of *tmm* SUs are clustered. In *tmm* siliques, stomatal initiation is suppressed, yet 18% of all SUs are clustered. These data suggest that the role of *tmm* in suppressing or enhancing SU initiation does not necessarily affect the role of this mutation in promoting clustering, i.e. both features can be uncoupled.

The *tmm* SUs that are clustered are not all of the same size. Cluster size (S) was measured as the average number of guard cells in all clustered SUs (Table 3).

Table 2. Frequency of clustered stomatal units^a

Organ	WT	<i>flp</i>	<i>tmm</i>	Predicted <i>tmm/flp</i> ^b	Observed <i>tmm/flp</i>	True? ^c
Anther	3 ± 1	49 ± 4	22 ± 3	62	36 ± 2	No
Sepal, Ab.	0	34 ± 2	7 ± 1	39	45 ± 3	No
Sepal, Ad.	0	26 ± 4	NA ^d	NA	NA	NA
Silique	0.4 ± 0.2	3 ± 1	18 ± 2	NA	14 ± 3	NA
Fl. stalk	0.4 ± 0.2	1.5 ± 0.5	14 ± 1	NA	15 ± 0.5	NA
Infl. stem	0.4 ± 0.2	3 ± 0.5	NA	NA	NA	NA
Caul., Ab.	0.1 ± 0.1	10 ± 2	13 ± 3	22	11 ± 2	No
Caul., Ad.	0.2 ± 0.1	14 ± 3	14 ± 3	26	24 ± 8	Yes
Cotyl., Ab.	0.5 ± 0.3	22	69	76	54	No
Cotyl., Ad.	0	33	24	49	21	No

^aCluster frequency (F%) ± SE; *n* = 15–20 for each organ

^bPredicted values for the double mutant were calculated from the union of the single-mutant values, $F_{if} = F_t + F_f - (F_t \times F_f)$, where F_{if} = F of *tmm/flp*, F_t = F of *tmm*, F_f = F of *flp*

^cDifferences between expected and observed values were determined by a chi-squared test ($P < 0.05$). A “yes” indicates that the observed result matches the prediction

^dNA = not applicable due to small numbers or lack of stomata

Ab. = abaxial, Ad. = adaxial, Caul. = cauline leaf, Cotyl. = cotyledon, Fl. stalk = flower stalk, Infl. stem = inflorescence stem

Table 3. Mean cluster size (S)^a

Organ	WT ^b	<i>flp</i>	<i>tmm</i>	<i>tmm/flp</i>
Anther	4	4.0 ± 0.1	5.3 ± 0.3	5.3 ± 0.2
Sepal, Ab.	NA ^c	3.9 ± 0.3	4	4.04 ± 0.03
Sepal, Ad.	NA ^c	4	NA ^c	NA ^c
Silique	4	4	4.6 ± 0.2	4.17 ± 0.05 ^d
Infl. stem	4	4	NA ^c	NA ^c
Caul., Ab.	4	3.92 ± 0.05	4.3 ± 0.2	4.04 ± 0.04 ^d
Caul., Ad.	4	3.74 ± 0.05	4.6 ± 0.4	4.1 ± 0.1 ^d
Cotyl., Ab.	4	4.3 ± 0.3	9 ± 1.4	5.8 ± 0.6 ^d
Cotyl., Ad.	4	4.14 ± 0.2	4.9 ± 0.2	4.3 ± 0.1 ^d

^aAverage number of guard cells for all clustered SUs ± SE; *n* = 15–20 for each organ

^bCluster size is reported for the few (0.6%) naturally occurring clusters in the WT

^cNA = not applicable due to the lack of clusters or stomata

^dDouble mutant is significantly smaller than *tmm* ($P < 0.05$)

Ab. = abaxial, Ad. = adaxial, Caul. = cauline leaf, Cotyl. = cotyledon, Fl. stalk = flower stalk, Infl. stem = inflorescence stem

Clusters in *tmm* are largest in the cotyledon and anther, while only small clusters occur elsewhere in the plant. In the abaxial side of the cotyledon, clusters are larger and occur more frequently in the basal than in the apical region of the blade (data not shown). When F is plotted against S for all *tmm* organs, there is a strong correlation between the two (Fig. 6). This indicates that the clustering effect of the *tmm* mutation regulates both the cluster frequency and size.

Dorsiventral and cylindrical organs have different cluster frequencies in flp. The frequency of clustering in *four lips* organs varies from 1.5% to 49% (Table 2). Most dorsiventral organs such as cotyledons, sepals, and anthers have higher cluster frequencies ($F = 20$ –50%) than cylindrical organs such as stems, flower stalks, and siliques ($F = 1$ –3%). But all *flp* organs have cluster frequencies that are higher than the WT ($F = 0.6\%$).

The *flp* organs with different cluster frequencies also differ in epidermal cell morphology. Most mature dorsiventral organs (such as leaves) have jigsaw-shaped pavement cells with wavy anticlinal walls, whereas cylindrical organs (such as stems) tend to have rectan-

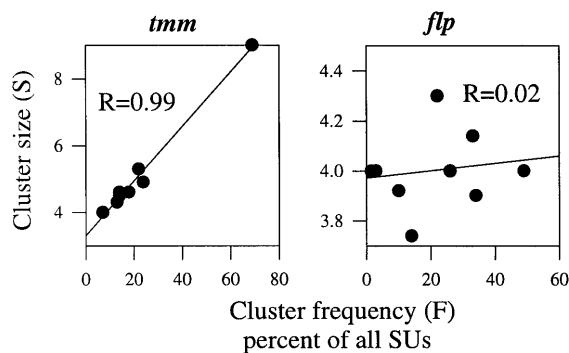


Fig. 6. Cluster size (S) and cluster frequency (F) in all organs examined are strongly correlated in *tmm* (A), but not in *flp* (B). Data points represent the average S and F for each different *Arabidopsis* organ examined (Tables 2, 3). *R* = correlation coefficient from regression analysis

gular pavement cells (Fig. 7A,C,E). The term pavement cell here refers to all shoot epidermal cells that are not differentiated as stomata or trichomes.

Mature cauline leaves have both types of pavement cells, with rectangular cells at the base of the lamina and

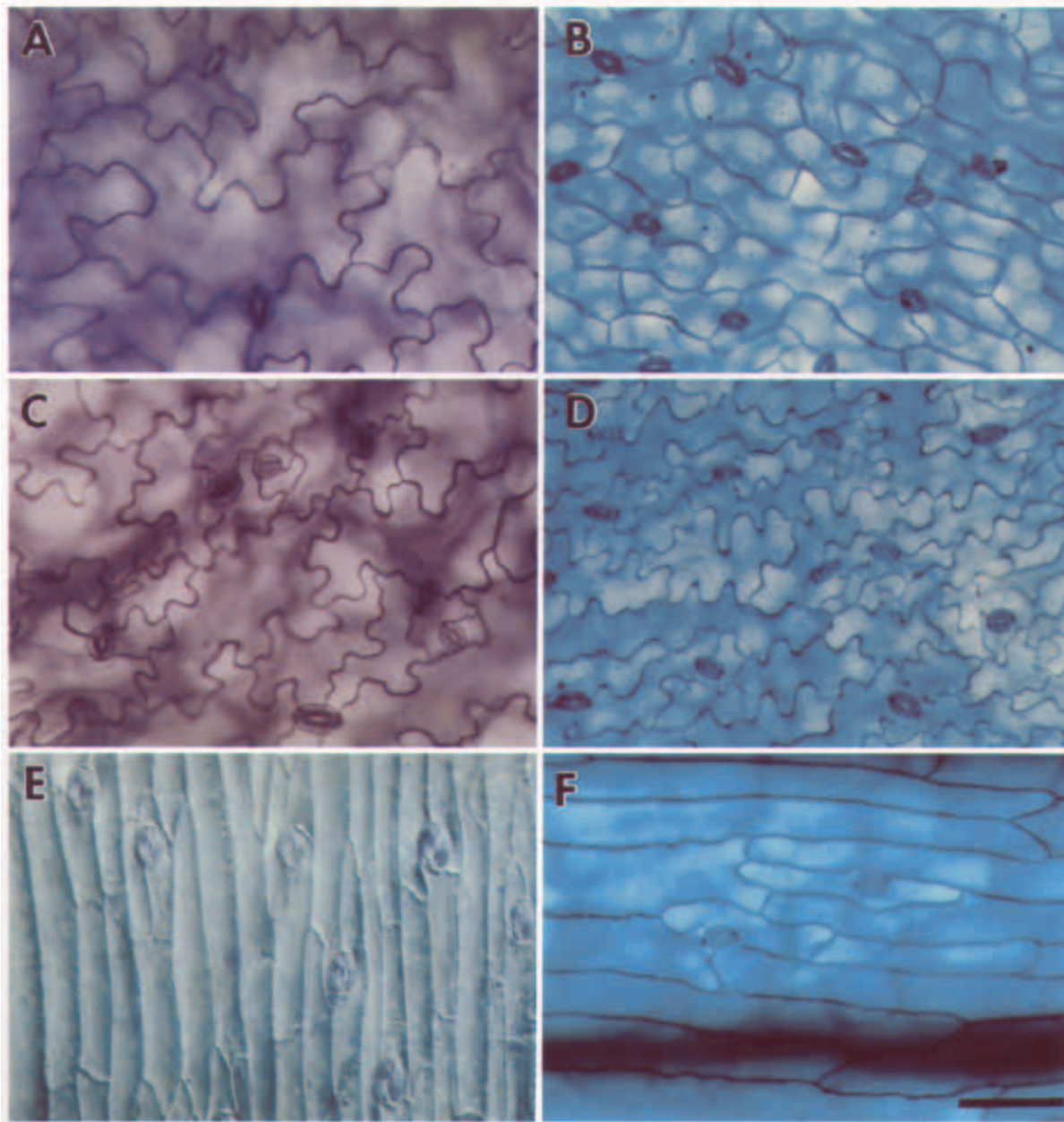


Fig. 7A–F. Different shapes of epidermal pavement cells in WT *Arabidopsis* plants. Jigsaw-puzzle-shaped cells are present in the cotyledons (A, C) and on the abaxial side of the cauline leaf (D). Narrow rectangular cells are present in the inflorescence stem (E) and at the base of cauline leaves (F). Adaxial cells in the cotyledon (A) are much larger than abaxial ones (C), while cauline leaf adaxial cells (B) are roughly the same size as abaxial ones (D). Bar = 30 μ m

jigsaw cells at the apex (Fig. 7B,D,F), with transitional forms located in between. Cauline leaves of *flp* have stomatal cluster frequencies that are intermediate between those for dorsiventral and cylindrical organs. There were no obvious differences in cluster frequencies between apical and basal regions of cauline leaves in *flp*, but these regions have not yet been sampled separately for quantification. Based on qualitative evaluation, all the above features also appear to be present in the three other alleles of *flp*.

Stomatal clusters in *flp* have a characteristic morphology which distinguishes them from *tmm* clusters. This includes the presence of some unpaired guard cells as well as a smaller cluster size. Unlike cluster frequency, cluster size in *flp* tends to fall within a narrow range, from a mean of 3.7 to 4.3 guard cells per cluster (Table 3), and cluster frequency is not correlated with cluster size (Fig. 6).

Relationships between flp and tmm in the double mutant

Total number of SUs. The distribution of stomata in the *tmm-1/flp-1* double mutant closely resembles that in *tmm*. Thus regions that lack all stomata in *tmm* also lack stomata in the double mutant. Similarly, the total number of SUs in *tmm* and the double mutant are

comparable in some organs where there is an enhancement (abaxial sepal, cotyledon, anther) or suppression (cauline leaf) of stomatal formation (Table 1). In these instances, the *tmm* mutation is clearly epistatic to *flp*.

In other cases, the total number of SUs in the double mutant is different from that in *tmm*. The general distribution of stomata in flower stalks and siliques of the double mutant resembles that for *tmm*. The total number of SUs in both these organs is significantly higher in the double mutant than in *tmm* even though these organs were of the same size (data not shown). On the adaxial surface of the cotyledon (which has an enhanced number of SUs in *tmm* relative to the WT), the double mutant has fewer SUs than the WT. In these organs, the presence of the *flp* mutation has moderated (silique, flower stalk) or even reversed (adaxial cotyledon) the effects of *tmm*. Thus in these organs and regions, there appears to be an interaction between the *flp* and *tmm* mutations.

Clustering frequency (*F*). In many organs the clustering frequencies of the double mutant were different from those of either mutant alone (Table 2). Since neither *tmm* nor *flp* have clustering in all SUs, the alleles for both mutations could be leaky, and some SUs could resemble the WT due to chance. If so, and if the mutations did not interact, then the fraction of clustered SUs in the double mutant should be predictable based on the single-mutant frequencies. This was tested quantitatively by comparing the union of the two single-mutant cluster frequencies to the double mutant (Table 2). In all cases (except the adaxial cauline leaf), the observed values for the double mutant were significantly different from the predicted values, indicating that the relationship between these mutations is neither epistatic nor additive in determining the frequency of clusters. The finding that actual *F* values in the double mutant deviate from the predicted values is consistent with the idea that neither mutation affects stomata at random.

Cluster size (*S*). In many organs, the clusters in the double mutant are smaller than those in *tmm* and larger than those in *flp* (Table 3). Additionally, there are fewer large clusters in the double mutant than in *tmm* (data not shown). This indicates that in some organs, the number of adjacent guard cells produced results from an interaction of both mutations in the double mutant.

Discussion

The *too many mouths* mutation affects stomatal formation in two important aspects of development and does so in a region-specific manner. In contrast, the *four lips* mutation appears to act downstream of *tmm* and affects dorsiventrally flattened and cylindrical organs differentially. The phenotype is consistent for each mutation both from plant to plant and for all known alleles. With the exception of smaller siliques and flower stalks in *tmm* (which may be due physio-

logically to the loss of stomata), neither mutation appears to affect any morphological trait other than stomatal development.

Regional up or down interactive regulation of stomatal initiation by *TMM*. Examination of the *too many mouths* phenotype in different *Arabidopsis* organs reveals that many regions exhibit stomatal suppression and upregulation, not just the cotyledon and inflorescence stem. In addition novel patterns were found such as gradients in one organ (flower stalk) or complete suppression and upregulation on opposite sides of the same organ (sepal). These results reinforce and extend previous observations that the *TMM* gene product can act as either a negative or a positive regulator of stomatal initiation depending on organ and region.

Although the *TMM* gene product is required for stomatal initiation in some organs and regions, in other locations it is not essential since many *tmm* organs do have stomata. In these organs, another unknown gene(s) may initiate stomatal formation. It is therefore plausible that at least two genes are necessary for stomatal formation throughout the plant, one of which is *TMM*. The *tmm* phenotypes of excess or too few stomata might result from a breakdown in reciprocal regulation between *TMM* and its hypothetical partner(s). In this model, *TMM* acts as a moderator of stomatal initiation; thus *TMM* would act as a negative or positive regulator of the formation of stomatal precursors depending upon interactions with its partner(s) and upon the regional context, perhaps even in a network of genes.

Some of these features may be analogous to the establishment by *bicoid* of the regional expression of downstream gap genes in *Drosophila* where the resulting interactions (including reciprocal regulation) within a network of genes (gap, pair-rule and homeotic) result in segment specification (Johnston and Nüsslein-Volhard 1992; Burstein 1995). These models contrast with the more or less linear pathway proposed for trichome specification in *Arabidopsis* where both the *TTG* and *GLI* gene products normally act as positive regulators (Hülkamp et al. 1994; Larkin et al. 1997). Differences in the complexity of regulation of the initiation of stomata and of trichomes might be expected since stomata, unlike trichomes, are essential for plant survival.

With respect to stomatal meristemoid initiation, *tmm* is usually epistatic to *flp*, since *flp* has no effect on SU number either alone or in a double mutant with *tmm*. However, in two organs, the silique and flower stalk, the total number of SUs in the double mutant is intermediate between the values for the two mutations separately, and adaxial cotyledons have fewer stomata than either single mutant. This suggests that in some cases, *FLP* might feed back to an upstream gene (*TMM*) to weakly suppress or enhance stomatal initiation that is promoted by *TMM*. Such feedback might be too weak to be detected when *FLP* alone is defective but could be revealed in the presence of a non-functional *TMM* gene product.

The *tmm* mutation is also valuable in that it reveals and is a marker for sub-organ regions not otherwise demarcated, such as the base of the flower stalk or the tips of the siliques. These features may help identify other region-specific genes and should provide regional markers for analyzing other developmental mutants.

Effects of tmm on stomatal initiation and patterning can be uncoupled. The extent of precursor cell formation (initiation) does not always correlate with the degree of stomatal cluster formation (patterning) in *tmm*. For example, abaxial *tmm* sepals have many more stomata than the WT but relatively few clusters, suggesting that initiation and patterning are separable events. Since stomatal patterning involves several variable components (Larkin et al. 1997), this uncoupling in *tmm* may reflect differences in how stomata are patterned in different regions.

The close correlation between cluster frequency and size in *tmm* suggests that both traits may result from the disruption of the same developmental process. The absence of a correlation between cluster frequency and size in *flp* reinforces the idea that the mechanisms of cluster formation are different in *flp* and *tmm*.

Clustering frequencies correlate with organ class in flp. The clustering phenotype of *flp* is also regulated in an organ-specific manner. Clustering frequency falls into two classes, strong (20–40%) clustering in dorsiventral organs, and weak (1–3%) clustering in cylindrical organs. In both classes, the size of the cluster is the same, but the “decision” to produce a cluster instead of a normal stoma is affected. Since the carpels that make up the silique are probably foliar in evolutionary origin, the reduced clustering in cylindrical organs compared to dorsiventral organs may not be simply due to differences between stems and leaves. All cylindrical organs in *Arabidopsis* have rectangular, elongated epidermal pavement cells that are arranged in files, whereas dorsiventral organs have jigsaw-shaped pavement cells. Perhaps the developmental program which determines pavement cell shape also influences the effect of the *flp* mutation. Expression of the *flp* phenotype might also vary with organ-level differences in the planes, frequency and timing of cell divisions.

The tmm and flp mutations appear to interact in determining cluster frequency and size. With respect to clustering, both genes appear to interact in most organs and regions. Cluster frequency and size are much lower in the double mutant than would be predicted by a simple additive effect of the two mutants. If the two mutations act independently, then cluster frequency would be at least equal to the union of the two mutant single values. Because the cluster frequencies of four different organs of the double mutant (Table 2) are much lower than the union of the single values, it is likely that stomata are not affected randomly by each mutation. This could result if a subpopulation of stomata is patterned normally in *flp* and *tmm*. *Sinapis*, a relative of *Arabidopsis* has been shown to contain

subpopulations of stomata with different developmental origins based on when meristemoids form (Landré 1972).

In the case of cluster size, the number of stomata in a cluster in the double mutant should be equal to the product or at least to the sum of the two single mutant values if the mutations contribute independently to clustering. Instead, it was found that in all organs, the cluster size in the double mutant was equal to or smaller than in *tmm*. In the latter case, the *flp* mutation apparently restrains the effect of the *tmm* mutation. Since cluster frequency and size are tightly coupled in *tmm*, it is possible that reductions in cluster size in the double mutant result from coupling to reductions in cluster frequency. Hopefully, developmental studies in progress will clarify whether there are stomatal subpopulations unaffected by these mutations and how clusters actually form.

Conclusions. The *TMM* gene product appears to control stomatal development in at least two ways. It regulates the initiation of the precursor cell and it ensures correct stomatal patterning. Loss of the latter function results in stomatal clustering. Loss of the former function results in varied effects ranging from increased production of stomata to their virtual elimination from specific organs and regions. Moreover, *TMM* may interact with other regulators of stomatal initiation and with markers that establish the local developmental context (region) for these interactions.

The *FLP* gene product also prevents clustering, but does not itself appear to be responsible for meristemoid initiation and it probably acts downstream of *TMM*. However, both gene products appear to interact in regulating cluster formation and in controlling initiation in a few organs. Overall, these results suggest that the regulation of stomatal development involves as yet unknown loci responsible for stomatal initiation as well as factors that confer regional identity.

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