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ISOLATION AND CHARACTERIZATION OF STOMATAL AND CYTOKINESIS DEFECTIVE MUTANTS IN ARABIDOPSIS

A DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

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1996

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To my parents, my wife and my sons.
ACKNOWLEDGMENT

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INTRODUCTION

The analysis of mutants has proved to be one of the most powerful approaches to studying the normal functions of corresponding wild-type genes. Many key developmental genes have been identified by mutant analysis and this type of research is often the foundation for much subsequent study. The work presented in this dissertation attempts to provide new insight into plant cell development by reporting the results of screening for and characterizing morphological mutants of stomata.

The effectiveness of a search for mutant cell phenotypes depends upon the sensitivity and specificity of the screen. Since a mutation might change the cellular phenotype without noticeably affecting the morphology of the organ, screening at an organ level would probably not be a sensitive screen for cellular mutants. Furthermore, different cellular defects could cause similar phenotypes at an organ level, complicating the analysis of gene function. For example, the 150 embryo mutant loci reported in *Arabidopsis* are likely to be a mixture of unrelated mutations of which at least some might only secondarily affect embryogenesis (e.g., Franzmann, et al., 1995). Indeed, the mutation *emb30* of *Arabidopsis*, originally isolated as an embryo mutation, seems to primarily affect cell division, elongation and adhesion, and only secondarily affects the development of the embryo (Mayer, et al., 1993; Shevell et al., 1994). Thus, it is
important to screen directly for mutants with an abnormal cellular morphology to understand the mechanisms underlying cell development.

Screening for cellular mutants is labor intensive since samples must be viewed with a microscope or with a dissecting scope. Nevertheless, many valuable cellular mutants have been found in animals and plants, and their characterization has generated exciting information about the determination of cell fate, and about cell differentiation and cell division.

One such system has been bristle development in *Drosophila* where cellular mutants have been well studied by genetics, molecular and cell biology. Since so much is known about bristle formation and since it has parallels to stomatal formation, highlights of its development are reviewed here.

*A Drosophila* bristle is derived from a sensory organ precursor (SOP) cell, which is first selected from a proneural cluster of cells all of which have the potential to adopt the SOP fate. But once the SOP cell is selected, all the rest of the cells of the cluster adopt an epidermal cell fate. The SOP cell divides to give rise to four types of cells, the socket cell, shaft cell, sheath cell and neuron, which altogether form a bristle. The neuron is the end product of the SOP cell lineage and the neuron is functionally the most important cell type of the bristle (Hartenstein and Posakony, 1989).

It has been shown that a few positive and negative regulators function throughout the bristle developmental pathway, from the selection of the early precursor cell to the determination of the final cell type (Campos-Ortega, 1993; Posakony, 1994). Loss of gene function results in the altered production of both precursor cells and progeny cells.
For example, Notch and Delta are neurogenic genes which appear to be a receptor and ligand in the cell membrane. Null mutations at these loci cause more proneural cells to adopt the precursor cell fate, and all four progeny cells of the precursor cell adopt the primary fate and develop into neurons. Thus, many more clustered neurons instead of bristles are produced.

Bristle development parallels stomatal development in plants in that both concern pattern formation in the epidermis, both rely on precursor cell selection and determination, and both types of precursor cells divide asymmetrically and produce the key cell type, neurons or stomata, in the last division of the precursor cell.

In contrast to model animal systems such as Drosophila, our understanding of the mechanisms regulating cellular morphogenesis in plants is in its infancy, although some details are starting to emerge. Many mutations have been described affecting trichome and root hair determination and differentiation in Arabidopsis, and in a few cases the genes have been characterized, and thought to encode transcription factors (Marks and Feldmann, 1989; Herman and Marks, 1989; Schiefelbein and Somerville, 1990; Hülskamp et al., 1994; Larkin et al., 1994; Masucci and Schiefelbein, 1994; Rerie et al., 1994). In spite of these advances, we are largely ignorant of the genetic factors which control the formation of other cell types such as guard cells.

This dissertation reflects one effort towards finding morphological stomatal mutants, an effort which exploited the relative accessibility of epidermal cells for screening and the specialized and unique morphology of stomata. Here I report the isolation and characterization of three cellular mutants in the model plant Arabidopsis thaliana. Two
of them, *too many mouths* (*tmm*) and *four lips* (*flp*), appear to specifically affect stomatal development and are described in Chapter I. The third mutant, *cytokinesis defective* (*cyd*), affects the cytokinesis of several cell types in addition to stomata, and its phenotype is described in Chapter II.
CHAPTER I

THE MUTATIONS *too many mouths* AND *four lips* AFFECT STOMATAL DEVELOPMENT IN *ARABIDOPSIS*

Stomatal guard cells are a unique cell type and are found on most aerial surfaces of plants. In dicots, the two guard cells are usually kidney-shaped and enclose the stomatal pore (Fig. 1). Stomata are crucial for plant life because they control gas exchange for photosynthesis by adjusting the width of the pore in response to various signals such as light, and the concentrations of CO₂ and abscisic acid in the leaf (Raschke, 1979).

Stomata are usually distributed in a characteristic pattern, featuring the presence of a stoma-free region surrounding each stoma (Sachs, 1991). This spacing pattern appears to be developmentally regulated because the occurrence of adjacent stomata is much rarer than it would be in a random distribution.

The pattern and distribution of stomata in many dicotyledonous plants derives from the formation and developmental behavior of meristemoids, the stomatal precursor cells. Meristemoids were originally defined as cells that continue to divide after most surrounding cells have stopped dividing, and like meristematic cells, they are usually small and cytoplasmically dense (Bünning, 1953; Fig. 1). A meristemoid forms through the asymmetric division of a protodermal cell. Most meristemoids then undergo one to
several asymmetric divisions. The larger progeny cell that results from each division differentiates into a non-stomatal epidermal cell (EC). The smaller progeny cell may continue to act as a meristemoid and divide asymmetrically. After the last EC is produced, the meristemoid becomes a guard mother cell which undergoes a terminal symmetric division to produce two guard cells (Sachs, 1991). In addition to this general scheme of stomatal formation, Sachs and Novoplansky (1993) demonstrated in *Peperomia* that stomata can also form directly from meristemoids that do not produce any ECs. Thus, meristemoids can be precursors only of stomata, or of both stomata and some ECs.

Stomatal development in *Arabidopsis* has not been described in detail. In the Brassicaceae, a family which includes *Arabidopsis*, there are usually 2-3 ECs neighboring each stoma and they are progeny cells of the same meristemoid that produces the stoma (Pant and Kidwai, 1967; Paliwal, 1967; Landré, 1972). Meristemoids are present in *Arabidopsis* (Wei, et al., 1994) and probably act in a way similar to that in other members of the family. Telfer and Poethig (1994) found that the majority of stomata in *Arabidopsis* are anisocytic, i.e., three ECs surround each stoma and one of the them is usually much smaller. However, besides size differences between neighbor cells surrounding some stomata, there appears no other reliable morphological feature to distinguish subsidiary cells.

Despite their morphological uniqueness, physiological importance, and well-studied differentiation (Sack, 1987), little is known about the mechanisms that underlie stomatal patterning, determination, and differentiation. To our knowledge, besides the barley mutant *eceriferum-g* that produces a small proportion of double and triple stomata
complexes (Zeiger and Stebbin, 1972), no other genes that regulate stomatal development have been identified. In contrast, in *Drosophila* bristle development which is also concerned with pattern formation in the epidermis, molecular, cellular and genetic studies have generated substantial evidence providing significant mechanistic insight into cell fate determination and pattern formation (Campos-Ortega, 1993; Posakony, 1994).

To understand how stomatal patterning, determination, and differentiation are regulated, a screen for morphological stomatal mutants was carried out with ethyl methane-sulfonate-mutagenized M$_2$ seeds of *Arabidopsis* using light microscopy. This chapter describes the isolation and characterization of two stomatal mutants, *too many mouths* (*tmm*) and *four lips* (*flp*), that have extra adjacent stomata. *tmm* influences the production of both guard mother cells and meristemoids. *flp* also influences the production of guard mother cells and possibly meristemoids. The phenotypes of these mutants also differ in the spatial arrangement of clustered stomata and in the presence of unpaired guard cells found only in *flp*. 
MATERIALS AND METHODS

Plant Material and Culture

Ethyl methane sulfonate-mutagenized \(M_2\) seeds of \textit{Arabidopsis thaliana} Columbia ecotype (Lehle Seeds, Round Rock, TX) were used for screening mutants. \(M_1\) seeds were imbibed in 0.13\% ethyl methane-sulfonate for 10 hours at 23°C. To facilitate screening and mapping using morphological markers, the seed line used lacked trichomes because it was homozygous for the \textit{glabrous1 (gll)} mutation (Marks and Feldmann, 1989). For screening, \(M_2\) seeds were surface sterilized by soaking in 30\% (v/v) Clorox for 10 minutes, washed 5 times with sterilized distilled water, and then sown on 1\% agar containing the nutrients described by Haughn and Somerville (1986) plus 2\% sucrose. Gridded square dishes containing the seeds were sealed with porous surgical tape (Micropore, 3M Corp.) to allow some gas exchange. Seedlings were grown under continuous 50-100 \(\mu\text{mol m}^{-2} \text{sec}^{-1}\) photosynthetically active radiation provided by cool-white fluorescent tubes and maintained at approximately 22°C. To allow seed set of weak putative mutants, seedlings were transferred to sterile Magenta GA7 boxes (Sigma) filled with the same medium. For gene mapping, seedlings were grown in Magenta boxes under the same conditions in order to harvest the whole seedling for DNA isolation and to reduce microbial contamination. For crosses and phenotypic characterization, plants were grown in pots in a soil mix that
included peat, perlite, and vermiculite under similar temperature and light intensity.

**Mutant Screening**

Cotyledons were screened for mutant phenotypes when the seedlings were 12-20 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." The "peels" were obtained by pressing the cotyledon onto clear double-stick tape affixed to a microscope slide and then scraping off the bulk of the cotyledon using a razor blade. The surface of the cotyledon that was stuck to the tape needed to be dry for optimal removal of the bulk of the cotyledon. The resulting abaxial epidermis was then stained with 0.05% (w/v) toluidine blue in 0.05% (w/v) borate or with 0.05% (w/v) crystal violet. The stomatal phenotype was evaluated at 400X magnification using bright-field microscopy. Over 21,000 M₂ seedlings from ~7,000 parental (M₁) plants were screened.

**Genetic Analysis**

Crosses were made between mutant and wild-type plants (backcrosses), and between *tmm* and *flp*. Each mutant was backcrossed to the wild type three times. Phenotypes of the F₁ and F₂ plants were evaluated and scored. A putative double mutant was selected by the presence of a new phenotype in some of the F₂ plants from the cross of *tmm* and *flp*. The double mutant was then confirmed by separately crossing the putative double mutant plants with each single mutant parent and evaluating the phenotypes of all the F₁ plants.
Mapping of the Mutations

Mapping of the mutations was first attempted by separately crossing \textit{tmm} and \textit{flp} with the morphological marker line DP24 (obtained from \textit{Arabidopsis} Biological Resource Center at Ohio State University). DP24 contains the markers \textit{erecta-I} (\textit{er-1}, chromosome 2), \textit{brevipedicellus-I} (\textit{bp-1}, chromosome 4), \textit{transparent testa glabra-I} (\textit{ttg-1}, chromosome 5) and \textit{yellow inflorescence-I} (\textit{yi-1}, chromosome 5). Because the mutants were in a \textit{gll} (chromosome 3) background, a total of 5 markers on chromosomes 2 to 5 were used in the mapping. However, analysis of the two F\textsubscript{2} populations did not reveal linkage of either \textit{tmm} or \textit{flp} to any of the markers (data not shown). Therefore, Cleaved Amplified Polymorphic Sequence (CAPS) markers (Konieczny and Ausubel, 1993) were used to continue the mapping.

For CAPS mapping, the double mutant of \textit{tmm} and \textit{flp} was crossed with Landsberg \textit{ERECTA} plants and then F\textsubscript{2} populations were generated. F\textsubscript{2} plants with a single mutant or a double mutant phenotype were selected. DNA was then isolated from individual F\textsubscript{2} plants or from pooled F\textsubscript{3} plants derived from a single parent, by the DNA minipreparation method (Dellaporta et al., 1983). Other procedures were as described by Konieczny and Ausubel (1993). Based on the mapping results with the morphological markers, CAPS markers on chromosome 1 were the first to be tested, and indeed, linkages were found for both mutations. All the primers used for polymerase chain reaction (PCR) amplification were a gift from Dr. Roger Hangarter and were as described by Konieczny and Ausubel (1993), except that the primers for marker PAB5, whose sequences were obtained by a computer search (\textit{Arabidopsis} Research Companion, Massachusetts General
Hospital/Harward), were independently synthesized (DNAGENCY, Aston, PA). The protocols for DNA isolation, PCR amplification, enzyme digestion and agarose gel analysis of the final DNA products are described more fully in the Appendix of this dissertation.

Quantitative Characterization of Phenotypes

Quantitative data were collected from independent experiments under various culture conditions, including 50 µmol m⁻² s⁻¹ or 100 µmol m⁻² s⁻¹ light intensity and pot culture, or 50 µmol m⁻² s⁻¹ and culture on supplemented agar medium. For experiments in pots, 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 ECs were determined in each of seven randomly selected fields per cotyledon. Each field represented 0.03 mm² of the cotyledon surface. For scoring purposes, an SU was defined as all those stomata or guard cells actually in contact with one another. Some mutant SUs consisted of multiple stomata or more than two guard cells. Other SUs consisted of a single stoma or a single isolated guard cell. All (non-stomatal) ECs were scored together, regardless of whether they were or were not in contact with guard cells.

Cotyledon area was determined from tracings on a video monitor (60X total magnification) made using a Nikon dissecting scope (SMZ-U) coupled to a video camera. Areas were analyzed either with an area meter (model LI-3100, LICOR, Inc., Lincoln, NE), or a graphics tablet and SigmaScan software. Cotyledon area and parameter
densities were used to calculate the total (absolute) numbers per cotyledon of stomata, SUs and ECs, and frequencies of SUs with different numbers of guard cells.

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

**Hand Sectioning**

Hand sectioning was carried out on a microscope slide under a dissecting microscope using a scalpel with a 2-3 mm cutting edge. This scalpel was made by first breaking a double-sided razor blade (Personna Super) into one-sided small angular pieces using a pliers, and then inserting each piece into the end of a wooden applicator stick. Paradermal slices were cut with the cotyledon or leaf sample held with either double-sided tape attached to a slide, or by forceps.

**Microscopy**

Light micrographs of epidermal peels or of hand sections of cotyledons 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 hours were also examined.
RESULTS

Stomata in Wild-type Cotyledons

Arabidopsis cotyledons are the first pair of leaf-like organs and they develop during embryogenesis. Upon germination and illumination, they become expanded and green. Both sides of the Arabidopsis cotyledon contain apparently functional stomata, and the abaxial epidermis has many more stomata than the adaxial side. Cotyledons were the primary organ used in screens for stomatal mutations. This chapter focuses on cotyledons, because the tmm phenotype is more strongly expressed in cotyledons than in leaves, and because their expansion is complete about 10 days after germination, a timing which expedites quantitative comparisons. The arrangement and development of stomata in the cotyledon appear similar to that in true leaves.

In wild-type cotyledons, stomata are separated by one or more non-stomatal ECs (Figs. 2A and 3A). Most, but not all, ECs adjoin guard cells. The number and size of ECs surrounding each stoma vary, but stomatal complexes are often anisocytic, with three ECs in contact with the stoma, and with one of these ECs being smaller (Figs. 1D, 2A and 3A). However, exceptions to the anisocytic configuration, such as four ECs around a stoma, occur fairly often (Fig. 2A). Because the number, arrangement and size of ECs surrounding each stoma vary, subsidiary cells, in the sense of morphologically
distinguishable ECs adjacent to stomata (Esau, 1977), are not universally present in *Arabidopsis*.

Meristemoids can be identified as small, cytoplasmically dense cells next to somewhat larger cells, and they usually have a triangular shape (Fig. 4A). The alignment of cell walls suggests that a meristemoid is produced by an earlier asymmetric division (Fig. 4A). Meristemoids in developing cotyledons seem to have a distribution and density related to those of stomata (Figs. 4A-D). Meristemoids or guard mother cells are also present in regions containing mature stomata (Figs. 4B-D), even in fully expanded cotyledons (Fig. 2A). Guard mother cells appear to be more rounded than meristemoids which tend to be angular (Figs. 4B-C).

Younger stomata are often found near older ones. This could result from the younger stoma forming from a so-called secondary meristemoid. It is known in the Brassicaceae that a neighbor cell of a existing stoma can produce a secondary meristemoid (Landré, 1972). Sometimes, sequential stages of stomatal development in nearby stomata could be observed (Fig. 4D), but more often stomatal development in *Arabidopsis* was asynchronous.

**too many mouths**

The most characteristic phenotype of *tmm* in cotyledons is clustered stomata (Figs. 2C and 3C). In the plants grown in soil under 100 μmol m⁻² s⁻¹ light intensity (Fig. 5A and Tables 1-2, Experiment 2), ~55% of the SUs contained more than one stoma. The remaining 45% of the SUs consisted of two guard cells and resembled wild-type stomata.
Depending upon the experiment, the largest cluster contained 26 (Fig. 5A) or even more guard cells. But approximately three out of four SUs that were clustered contained no more than eight guard cells. In *tmm*, SUs have only an even number of guard cells (Fig. 5 and Tables 2-3).

In developing or even fully expanded *tmm* cotyledons, the stomata within a cluster are often at different stages of development, and the stomata are often at angles with respect to each other so that the long axes of their pores are rarely parallel (Figs. 2C, 3C and 6A). The different stages of development among stomata within a cluster may result from the guard mother cells being produced sequentially. But stomata within a large cluster are usually not arranged simply from old to young, i.e., developing stomata can be adjacent to each other as well as to mature stomata (Figs. 6A and 7). In *tmm*, stomatal 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. The most commonly found arrest is that the stomatal pores never form or open developmentally (Fig. 6A). Some stomata in clusters have open pores that are smaller than normal. Stomata with unformed 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 (Fig. 7).

Developing *tmm* cotyledons were examined to detect earlier stages in cluster formation. In the cotyledon abaxial epidermis of four day old seedlings, meristemoids are
often found adjacent to developing stomata and guard mother cells (Fig. 8A). These cells can be recognized as meristemoids because they appear to be the smaller daughter cell of an asymmetric division and they resemble wild-type meristemoids in size and shape (Figs. 4A and 8A). The presence of a meristemoid next to a differentiating stoma was never seen in wild-type cotyledons of the same age. Based upon analysis of cell wall patterns in *tmm*, these meristemoids and their associated guard mother cells or developing stomata appear to be produced from a common cell lineage. This pattern of meristemoids in contact with developing stomata suggests how some clusters might be produced, and is consistent with the observation that stomata in mature clusters are often found in different stages of development. Although two or more adjacent stomata can be detected in the cotyledons of 4 day old *tmm* seedlings (Fig. 4B), there are many fewer clusters and they are much smaller compared to those in mature *tmm* cotyledons, indicating that the majority of clustered stomata are produced later in cotyledon development.

The phenotype of clustered stomata indicates that the *tmm* mutation increases the number of guard mother cells produced. The presence of extra stomata adjacent to other stomata also shows that the *tmm* mutation can disrupt 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*, these abnormalities could be a general problem of limited growth, perhaps due to stomatal crowding. Thus, the primary effect of this mutation may be on stomatal production rather than on stomatal differentiation.
**four lips**

The word "stoma" is derived from the Greek for "mouth," and so, by analogy, the guard cells are the "lips." The second stomatal mutant is named *four lips* because it has many stomatal clusters containing two adjacent stomata (four guard cells: Figs. 2B, 3B, 5A, 9A; Tables 2-3). Another distinctive feature of *flp* is the presence of a small proportion of unpaired guard cells, either single and isolated or part of a small cluster (Figs. 5B, 9B-D, Tables 2-3). Thus, unlike *tmm*, *flp* has SUs with either an even or an odd number of guard cells. Of all SUs scored (Experiment 2 in Tables 1-2), 21% consisted of four guard cells per SU, and ~8% consisted of one, three, five, six, seven, or eight guard cells per SU (Table 2). Altogether, ~29% of all SUs were abnormal and 71% were similar to wild-type stomata. The same general quantitative trends were seen in four other experiments (Experiments 1 and 3-5; Tables 1 and 3).

In some *flp* clusters, adjacent stomata are arranged side by side, i.e., the long axes of the guard cells or stomatal pores are more or less parallel (Figs. 2B, 3B, 9A-B and 9D), a situation which is rarely seen in *tmm*. Differentiation of guard cells is usually normal even in unpaired guard cells (Figs. 9B-D). However, pore development sometimes appears arrested, and some guard cells are abnormally shaped. Clusters in *flp* appear more constricted in overall expansion (Figs. 2B, 3B, 9A-B and 9D) than those in *tmm*, although the average cluster size in *flp* is much smaller.

The stomatal clusters of *flp*, as well as those of *tmm*, seem to be distributed in a pattern characteristic of the wild type, with each cluster (or single guard cell) positionally equivalent to a single stoma in the wild type (Figs. 2A-C and 3A-C).
Thus, like \textit{tmm}, \textit{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 \textit{flp} clusters are smaller and have a different morphology than \textit{tmm} clusters, and of the two, only \textit{flp} has unpaired guard cells.

Developing cotyledons of \textit{flp} were also examined to seek clues of how the clusters were formed. It appears that abnormal meristemoids committed to forming guard cells first divide symmetrically to produce what appear to be two guard mother cells. These guard mother cells can then divide normally (symmetrically) to produce two either laterally-aligned (Figs 10A) or two offset (Fig. 10D) stomata. Alternatively, the second round of division can be delayed, absent or asymmetrical (Figs. 10B-C). These events could produce clusters of three or four guard cells often found in mature \textit{flp} cotyledons. It is not clear whether these abnormal meristemoids previously produced ECs before they underwent the symmetric divisions.

\textbf{Genetic Analysis and Mapping of the Mutations}

Genetic analysis indicates that \textit{flp} and \textit{tmm} are separate loci because crosses between them yielded F$_1$ plants with a wild-type stomatal phenotype (Table 4). They are also nuclear recessive mutations because approximately one of four F$_2$ plants showed the single mutant phenotype following separate backcrosses of each mutant to the wild type. After three backcrosses, each mutant phenotype resembled that of the original M$_2$ isolate, indicating that it is unlikely that second mutations are responsible for the stomatal phenotypes. Only one allele has been found for each locus, \textit{tmm}1-1 and \textit{flp}1-1.
Both mutations were mapped to chromosome 1 using the PCR-based CAPS markers. Previous attempts to map the mutations using the external morphological marker line DP24 containing markers on chromosomes 2, 4 and 5, failed to reveal any linkage (the mutants contain g/U which was also scored as a marker for chromosome 3). But the morphological markers helped narrow the search to chromosome 1 for CAPS mapping, and indeed, the first test with CAPS markers on chromosome 1 established linkages. According to the CAPS data, \textit{tmm} is 8.6 cM from the marker ADH (35 $F_2$ plants with \textit{tmm} phenotype were tested for segregation for Columbia and Landsberg ecotype polymorphisms) and 18.1 cM from the marker PAB5 ($n = 36$). \textit{flp} is 3.0 cM from the marker NCC1 ($n = 33$) and 25.8 cM from the marker PVV4 ($n = 33$). Thus, \textit{tmm} and \textit{flp} map to the lower and upper arms of chromosome 1, respectively (Fig. 11).

\textbf{Double Mutant}

Following the cross between \textit{flp} and \textit{tmm}, the double mutant was identified in the $F_2$ generation by the presence of a new phenotype characterized by more and larger clusters than \textit{flp}, with SUs containing an even and odd number of guard cells (Figs. 2D, 3D, 6B and 9E).

After self-fertilization, this new phenotype was stable and present in all progeny for five generations. These putative double mutant plants were then backcrossed separately to \textit{tmm} or to \textit{flp}. Six independent crosses per mutant parent were performed. 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 \textit{flp} and
The double mutant had approximately the same frequency of clustering as did *tmm*, i.e., 53% of all SUs had clustered stomata, but cluster size was slightly smaller. The double mutant had fewer clusters with 20 or more guard cells (Figs. 5A-B) and fewer guard cells per SU or per cluster (Figs. 12A-B). The double mutant also differed from *tmm*, but resembled *flp*, in having some SUs with odd-numbered guard cells (Figs. 5B, 6B and 9E). 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 (Figs. 5A-B).

Cluster morphology in the double mutant had features of both parents. Like *tmm*, some stomata in the clusters were arranged in a spiral or irregular fashion so that the long axes of the stomata or guard cells were not parallel. But unlike *tmm*, some clustered stomata were positioned laterally (Fig. 3D), a feature also found in *flp*. Clusters in the double mutant tended to be more compact than in *tmm*, another feature found in *flp* (Figs. 2D and 3D). Compact clusters contained stomata that were arranged laterally, whereas looser clusters contained stomata positioned at various angles with respect to one another (Fig. 3D).

Thus, the phenotype of the double mutant in the cotyledon is neither identical to either mutant parent (epistasis) nor simply 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. These data suggest that both additivity and interaction of the mutations might occur, but it is hard to distinguish
which part(s) of the double phenotype is (are) due to addition or interaction of the mutations.

Developing cotyledons of the double mutant were also examined to reveal how the clusters are formed. Typical angular-shaped meristemoids were seldomly seen in the cotyledons. Instead, larger and rounder cells were frequently observed (Figs. 13A-D), cells that were either abnormal meristemoids or guard mother cells. As in tmm, the density of meristemoids appeared higher than that of the wild type (Figs. 4A-D and 13A-D) in the double mutant, and meristemoids or guard mother cells were often found adjacent to developing stomata (Figs. 13B and 13D). Some guard cells appeared to form directly from the smaller daughter cells produced by asymmetrical divisions of the abnormal meristemoids (Fig. 13B). Some of the adjacent abnormal meristemoids or guard mother cells were more or less equal in size (Fig. 13D).

Quantitative Analysis of Cotyledon Phenotypes

To assess how stomatal production was affected in cotyledons, the cotyledon areas, densities and absolute numbers of stomata, SUs, and ECs were determined. Plants were grown at either 50 or 100 μmol m$^{-2}$ s$^{-1}$ on soil or agar (see Table 1).

Cotyledon Area

The area of flp cotyledons was equal to or larger than that of the wild type (Table 1). The area of tmm cotyledons was equal to or smaller than that of the wild type (Table 1). tmm cotyledons were smaller than flp cotyledons. The area of cotyledons of the double mutant was variable (Table 1). Under the conditions tested, flp cotyledons tended
to have the largest area among the three mutant genotypes.

The cotyledon area varied under different growing conditions. It appeared to increase under higher light intensity or when grown on agar medium.

**Stomata**

As expected, all three mutant genotypes had more stomata than did the wild type under the conditions tested (Figs. 14-15). High light intensity appeared to increase stomatal production. Plants of the four genotypes grown on agar under 50 μmol m⁻² s⁻¹ appeared to have comparable numbers of stomata to plants grown on soil under 100 μmol m⁻² s⁻¹ (data not shown). Thus, both mutations positively influence stomatal production in the abaxial epidermis of the cotyledon.

**Stomatal Units**

Both *tmm* and the double mutant had more SUs on either an absolute or a density basis than did the wild type under the growing conditions tested (Figs. 14-15). Because each SU must be produced from at least one meristemoid, these data show that *tmm* and the double mutant produce more meristemoids in the cotyledon abaxial epidermis than does the wild type. Although not quantified, meristemoid density also appeared higher in developing *tmm* and the double mutant cotyledons (Figs. 4A-D, 8A-B and 13A-D).

In *flp*, the absolute number of SUs was greater than that of the wild type at the higher light intensity (Experiment 2, Table 1, Fig. 14), but it was the same as that of the wild type at the lower light intensity (Experiment 3, Table 1, Fig. 15). This difference correlates with cotyledon area (Table 1). The density of SUs in *flp* is the same as in the wild type under either of the two light conditions. Developing *flp* cotyledons also
appeared to have the same density of SUs as that of the wild type (Figs. 4A-D and 10A-D).

In another independent experiment from fully expanded cotyledons (22 days old), the total number and density of SUs between the wild type and \textit{flp} were not statistically different (Fig. 16). The two genotypes also had the same cotyledon area (Experiment 5, Table 1). But in this experiment, \textit{flp} cotyledons had more arrested meristemoids and arrested guard mother cells than the wild-type cotyledons (Fig. 16). These data suggest that the \textit{flp} mutation might result in a weak enhancement in meristemoid production.

\textit{Epidermal Cells}

Although cell size was not measured, there was no obvious difference in the area of ECs among the four genotypes. Both the absolute number and the density of ECs decreased in the double mutant compared to the wild type at the higher light intensity (Experiment 2, Table 1; Fig. 14). In the same experiment, the absolute number and the density of ECs in \textit{tmm} was not statistically different from those in the wild type. The absolute number, but not the density, of ECs in \textit{flp} was greater than that in the wild type.

At lower light intensity (Experiment 3, Table 1), the absolute number, but not the density of ECs in both \textit{tmm} and the double mutant was lower (Fig. 15) compared to the wild type, but both the density and absolute number of ECs were not statistically different between \textit{flp} and the wild type.

Despite these mixed results, when the data were expressed as a ratio, i.e., as the number of ECs per SU (Fig. 17), the means of the \textit{tmm} and the double mutant were consistently different from those of the wild type; both produced fewer ECs relative to
SUs, with the double mutant producing fewer ECs per SU than did *tmm*. *flp* had the same ratio of ECs to SUs as the wild type.

Thus, the *tmm* mutation upregulates the production of meristemoids and stomata, and downregulates the production of ECs in the cotyledon abaxial epidermis. The ratio of ECs to SUs in *flp* does not eliminate the possibility that *flp* might also slightly downregulate the production of ECs because the double mutant shows a stronger reduction in ECs than does *tmm*.

**Specificity of the Mutations**

*flp*, *tmm*, and the double mutant 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.

At a cellular level, no obvious pleiotropic effects of these mutations were found. For example, trichome formation and distribution seemed unaltered in plants homozygous for *GL1* in which the *flp* or *tmm* mutations were introgressed. Root hairs of either *tmm*, or *flp*, or the double mutant also appeared to be distributed in a normal pattern. Thus, among epidermal cell types, the mutations appear to be specific for stomatal production, although more work is needed to confirm that the mutations are not pleiotropic.

**Mutant Stomatal Phenotypes in Other Organs**

Various organs were examined using whole mounts, epidermal peels, and hand sections, to determine semi-quantitatively the extent of stomatal clustering throughout the
plant. In *tmm* and the double mutant, the clusters appear to be larger and more frequent in the abaxial epidermis of the cotyledon than in the rosette leaves. In *flp*, the percentage of abnormal SUs, mainly paired stomata, seems comparable between the cotyledon and all leaves.

In wild-type and *flp* plants, stomata are distributed throughout the inflorescence stem (data not shown). However, in *tmm* and the double mutant, stomata are absent or very rare in the inflorescence stem. In addition, the basal portion of the pedicel (flower stalk) in *tmm* and the double mutant is stomata-free. Between the apical and basal regions of the pedicel, there is a gradient in stomatal production with many stomata (some of them clustered) at the apical end, fewer stomata in the mid-length, and almost none at the base (data not shown).

Thus, in *tmm* and the double mutant, there is a proliferation of stomata and of meristemoids in the abaxial epidermis of the cotyledon, an almost complete suppression of stomatal formation in the inflorescence stem, and a partial, polarized suppression of stomatal formation in the pedicel.
DISCUSSION

tmm and flp are novel Arabidopsis mutations that affect stomatal development. They are both recessive at different loci on chromosome 1. Their most characteristic phenotype is extra adjacent stomata in the epidermis of several organs where single stomata are normally present. Both mutations influence stomatal production, possibly by altering precursor cell (meristemoid) activity. tmm also affects stomatal production by regulating meristemoid formation. tmm both up- and downregulates meristemoid production depending upon the organ or the organ domain. flp may also have a weak effect on meristemoid formation. Thus, both genes appear to be involved in stomatal formation at several developmental levels.

Stomatal Development in Arabidopsis

Stomatal development in Arabidopsis appears to follow the same pattern deduced for other members of the Brassicaceae (Fig. 1; Paliwal, 1967; Pant and Kidwai, 1967; Landré, 1972). Meristemoids are certainly present and appear to give rise to ECs and stomata (Figs. 4A-D; Wei et al., 1994). However, the reconstruction of cell lineage of the meristemoid solely from the examination of cell wall patterns is no substitute for the study of the same cells through time, for example, through the use of dental impression material.
Sachs and colleagues have successfully used dental impression materials to repeatedly replicate the same area of epidermis to study stomatal development through time (Sachs, 1991; Sachs and Novoplansky, 1993). They were able to reveal significant variability in the formation and activity of meristemoids, e.g., some meristemoids might directly form guard mother cells without first forming ECs, a variability which would not be detected by examining the pattern of mature cells. Thus, a full assessment of wild-type stomatal development will require more intensive study of cell lineages.

Nevertheless, it is likely that wild-type stomatal development in Arabidopsis can be divided into at least five stages (Fig. 1 and 4A-D): (1) the patterning and initiation of meristemoids; (2) one to several asymmetrical divisions of the meristemoids; (3) the conversion of the meristemoids to guard mother cells; (4) the symmetrical division of each guard mother cell into two young guard cells; and (5) the differentiation of the guard cells to form stomata. Our data indicate that TK4M and FLP gene products might act during several of these stages to influence stomatal production, possibly by regulating cell fates.

**flp and tmm Primarily Affect Stomatal Production**

Extra adjacent stomata are the major feature of the two mutations. This phenotype 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 (Figs. 14 and 15). As discussed below, it appears that the effects of these mutations on stomatal spacing and differentiation may be secondary to an effect on production.
Stomatal spacing is dramatically altered in both mutants because stomata can often be adjacent to one another. The stomatal clustering in these mutant violates the most orderly feature of wild-type stomatal spacing (Sachs, 1991), i.e., there is no stoma-free zone around many mutant stomata. Nevertheless, the presence of a stoma-free zone around stomatal clusters or units suggests that neither mutation affects primarily the patterning of meristemoids. The patterning of mutant SUs more or less resembles that of the wild type, with each cluster or single guard cell in the mutants positionally equivalent to a single stoma in the wild type. This implies that, whatever the mechanism, patterning is kept intact in the mutants. Thus, the presence of adjacent stomata, which usually do not exceed four stomata per SU in both mutants, might result from an overproduction of stomata from each meristemoid rather than from altered patterning of the meristemoids themselves. If so, neither TMM and FLP plays a direct role in stomatal patterning, and they only secondarily influence stomatal spacing and SU density.

Stomatal differentiation in both mutants is sometimes abnormal, including arrested pore development and distorted guard cells. These abnormalities are usually found in tmm in smaller stomata tightly packed in clusters. 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, instances of abnormal differentiation might be a consequence of stomatal crowding induced by stomatal overproduction, or from senescence of the cotyledon preventing the eventual formation of the pore. If so, neither TMM nor FLP plays a direct role in guard cell differentiation. Therefore, it is likely that both mutations affect primarily the production of stomata.
*tmm* May Alter the Fate of Cells Produced by Meristemoids

The fact that fewer ECs relative to SUs are produced in *tmm* cotyledons, suggests that 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 may occur earlier, producing extra guard mother cells which are substituted precociously for ECs. In this case, *tmm* would influence the fate of the cells produced by the meristemoid.

Clusters might also be formed by other mechanisms in *tmm*. For example, extra guard mother cells might also be produced for a more extended period than they are in the wild type. Also larger clusters might be formed from several meristemoids if the formation of intervening ECs were repressed, although this is unlikely for most of the clusters. Clusters could also result from a breakdown in lateral inhibition. The classical lateral inhibition hypothesis for stomatal spacing states that a meristemoid and/or guard mother cell produces some sort of field that prevents previously formed, nearby cells, regardless of their origin, from developing into stomata (Bünning, 1965). Distinguishing between these possibilities will probably require study of cell lineages in the same area through time. Present data are consistent with the possibility that *tmm* produces extra guard mother cells at least in part at expense of ECs.

*tmm* Also Affects Meristemoid Formation

Several lines of evidence show that the *tmm* mutation affects the production of meristemoids themselves. Cotyledons of *tmm* consistently have more SUs than do
cotyledons of the wild type. Because it is likely that each SU derives from at least one meristemoid, \textit{tmm} upregulates meristemoid formation in the cotyledon. In the \textit{tmm} inflorescence stem and the basal part of the pedicel, stomata, and probably meristemoids, are virtually absent, whereas in the wild type, stomata are distributed throughout these areas. Therefore, in the stem and the basal part of the pedicel, the \textit{tmm} mutation appears to prevent entry into the pathway leading to stomatal formation. Thus, \textit{tmm} influences both the entry into the stomatal developmental pathway as well as later guard mother cell formation.

Because \textit{tmm} is recessive and likely to be a loss-of-function mutation, the \textit{TMM} gene product can exert either negative regulation on meristemoid formation, such as in the abaxial epidermis of the cotyledon, or positive regulation, such as in the inflorescence stem and the basal part of the pedicel. More studies are needed to elucidate the mechanisms that underlie these opposite effects of \textit{TMM} in different organs and domains.

As mentioned, meristemoids in a \textit{tmm} cotyledon might produce extra guard mother cells at the expense of producing epidermal cells, because the ratio of ECs to SUs is lower in \textit{tmm} than in the wild type. But the extra meristemoids in \textit{tmm} cotyledons must also originate from other cells. Normally a meristemoid is formed from a protodermal cell. It is not clear how the extra meristemoids in \textit{tmm} are produced, but it is possible that they are also produced at the expense of EC production. If so, this could also explain why the absolute numbers of ECs in \textit{tmm} and the double mutant are equal to or less than the wild type.
Possible Stages of *flp* Action in Cluster Formation

In the wild type, after a meristemoid is committed to be a guard mother cell, it only divides once symmetrically to form two guard cells. This meristemoid activity is apparently altered in *flp*, because abnormal SUs with one or more guard cells appear to derive from the same meristemoids (Figures 10A-C). This could imply that meristemoids in *flp* either do not divide and produce a single, isolated guard cell, or that they undergo more than one round of cell divisions after they are committed to forming guard cells to form more than two guard cells.

It is not clear whether these events occur before or after the meristemoid has produced a full complement of ECs. The number of ECs in *flp* is comparable to or more than that in the wild type, which suggests either that extra guard mother cells are produced after EC production is complete, or that the increase in meristemoid number in *flp* (see below) compensates for the reduction in ECs due to the precocious formation of guard mother cells.

Although *flp* has the same or more SUs per cotyledon than the wild type depending upon the experiment (Figs. 14-16), it also can have more arrested meristemoids or guard mother cells (Fig. 16). Thus, *flp* might also weakly upregulate meristemoid formation in cotyledons.

Clearly, a determination of when both *flp* and *tmm* act requires more developmental study, and the isolation and characterization of additional alleles, since the severity of existing alleles is unknown.
Double Mutant

The relationship between the \textit{FLP} and \textit{TMM} gene products appear complex. In the pedicel and the inflorescence stem, \textit{tmm} appears epistatic to \textit{flp} in that both \textit{tmm} and the double mutant show a gradient of stomatal production along the pedicel and both lack stem stomata. But in the cotyledon, neither mutation is epistatic; the double mutant has features of both parents. Furthermore, the intermediate cluster size of the double mutant compared to those of the two mutants cannot be explained by simple addition of both mutant phenotypes.

This intermediate cluster size may be caused by the \textit{flp} mutation since \textit{flp} clusters are much smaller than those in \textit{tmm}. This intermediate cluster size may also indicate that the functions of \textit{FLP} and \textit{TMM} overlap at least partially. But it is not clear whether the intermediate cluster size is due to \textit{flp} being epistatic to \textit{tmm} during a specific phase of the development, or to interaction between the two mutations during stages when their functions overlap.

Clusters in the cotyledon in the double mutant can contain both compact and loose regions (Fig. 3D). Loose clusters are a feature of \textit{tmm} and compact clusters with laterally arranged stomata are characteristic of \textit{flp}. Thus, clusters in the double mutant retain morphological features of both mutant parents. It cannot be excluded that physical constraint might play a role in forming loose and compact clusters in \textit{tmm} and \textit{flp}, respectively. But the presence of both loose and compact regions in the double mutant clusters must be due, at least partially, to the mutations. The relationship between the two mutations will probably be clarified through analysis of the phenotypes of other alleles.
Parallels in Development between *Arabidopsis* Stomata and *Drosophila* Bristles

Stomatal development has some intriguing parallels with bristle development in *Drosophila* (see Introduction to this Dissertation). *tmm* is also similar to some bristle mutants such as *notch* and *delta* in that all these mutations alter (increases in the *tmm* cotyledon) the number of precursor cells (SOPs and meristemoids), as well as the number of the last key cells (neurons and stomata) produced from the precursor cells at the expense of other epidermal cells. These parallels are consistent with the possibility that the *TMM* gene product is an important regulator of stomatal formation.

Stem Stomata Appear Unnecessary

It remains to be determined whether stomata in clusters and pairs in leaves and in cotyledons are functional, and whether clustering affects gas exchange. However, it is already clear that the absence of stomata in the inflorescence stem in *tmm* and the double mutant still allows vigorous seed set, at least in laboratory grown, relatively unstressed plants. Thus, inflorescence stem stomata are not necessary for relatively high seed set in *Arabidopsis*.

Other Abnormalities and Mutants

Stomatal abnormalities similar to the phenotypes of *flp* and *tmm* have been found in wild-type plants of various genera (Dehnel, 1967; Pant and Kidwai, 1967; Inamdar et al., 1969). These abnormalities occur in much lower frequency than in *tmm*, and the clusters previously reported were never as large as some of the clusters found in *tmm*. It
is possible that these rare phenotypes (in wild-type plants) result from malfunctioning of homologs of TMM and FLP.

Some trichome and root hair mutant phenotypes in Arabidopsis also show extra adjacent cells. Clustered trichomes occur both in the Tryptichon mutant and when a trichome determination gene (GLABROUS1) is overexpressed in plants heterozygous for a strong transparent testa glabra (ttg) allele (Hülskamp et al., 1994; Larkin et al., 1994). Interestingly, ttg alone also causes clustered root hairs (Galway et al., 1994). Clustered root hairs also occur in the root hair mutant rhd6 (Masucci and Schiefelbein, 1994) and other ethylene or auxin related mutants such as ctrl, axr1 and etr1 (Dolan et al., 1994; Masucci and Schiefelbein, 1994). The developmental mechanisms producing clustering in these systems are unknown, although ethylene seems to be involved in the root hair phenotype.

I am 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 acting in cuticle production also affects stomatal development. Although possible pleiotropic effects of tmm and flp 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, which cause the phenotype of extra adjacent stomata. The genes appear to act in different ways, yet
both are 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 meristemoid formation. The $FLP$ gene might also weakly affect meristemoid
formation. Thus, $TMM$, and perhaps $FLP$, regulate stomatal production at several
developmental levels.
### Table 1. Cotyledon Area (mm²) of Different Genotypes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Wild type</th>
<th>flp</th>
<th>imm</th>
<th>Double mutant</th>
<th>Light intensity (µmol m⁻² s⁻¹)</th>
<th>Soil vs. agar</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>5.5 ± 0.2</td>
<td>7.1 ± 0.2</td>
<td>5.6 ± 0.2</td>
<td>6.2 ± 0.1</td>
<td>50</td>
<td>agar</td>
</tr>
<tr>
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<td>5.0 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>100</td>
<td>soil</td>
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<tr>
<td>3</td>
<td>4.0 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>50</td>
<td>soil</td>
</tr>
<tr>
<td>4</td>
<td>4.3 ± 0.1</td>
<td>5.6 ± 0.2</td>
<td>---</td>
<td>---</td>
<td>50</td>
<td>soil</td>
</tr>
<tr>
<td>5</td>
<td>5.5 ± 0.2</td>
<td>5.4 ± 0.2</td>
<td>---</td>
<td>---</td>
<td>50</td>
<td>soil</td>
</tr>
</tbody>
</table>

*a Sample sizes were 6-10 cotyledons (one from each plant) in Experiment 1 and 20-25 cotyledons (one from each plant) in experiments 2-5 for each genotype.

*b Growing conditions were either agar medium in enclosed petri dishes or soil in pots.

*c Mean ± Standard Error.

Statistical analysis (t-test at the 0.05 level) shows that: (1) the cotyledon area of flp was larger than (Experiments 1-2 and 4), or equal to (Experiments 3 and 5) that of the wild type; (2) the cotyledon area of imm was equal to (Experiments 1-2), or smaller than (Experiment 3) that of the wild type; (3) the cotyledon area of the double mutant was larger than (Experiment 1), or equal to (Experiment 2), or smaller than (Experiment 3) that of the wild type.
Table 2. Number of Stomatal Units with Different Number of Guard Cells per Cotyledon*

<table>
<thead>
<tr>
<th>No. of Guard Cells/SU</th>
<th>Wild type</th>
<th>flo</th>
<th>tmm</th>
<th>Double mutant</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>3±2</td>
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<tr>
<td>2</td>
<td>454±24b</td>
<td>369±17</td>
<td>271±26</td>
<td>284±33</td>
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<tr>
<td>3</td>
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<td></td>
<td></td>
<td>16±6</td>
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<tr>
<td>4</td>
<td>4±2</td>
<td>111±13</td>
<td>108±13</td>
<td>131±18</td>
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<td>29</td>
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<td>1±1</td>
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* Data from Experiment 2 described in Table 1.

b Mean ± Standard Error.

Shading represents clusters with an odd number of guard cells.
Table 3. Number of Stomatal Units with Different Number of Guard Cells per Cotyledon*  

<table>
<thead>
<tr>
<th>No. of Guard Cells/SU</th>
<th>Wild type</th>
<th><em>flp</em></th>
<th><em>lmm</em></th>
<th>Double mutant</th>
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<tbody>
<tr>
<td>1</td>
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<td></td>
<td></td>
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<tr>
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<td>240±17</td>
<td>238±20</td>
<td>311±39</td>
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<td>1±1</td>
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</table>

*Data from Experiment 3 described in Table 1.

bMean ± Standard Error.

Shading represents clusters with an odd number of guard cells.
Table 4. Genetic Analysis of *flp* and *tmm*

<table>
<thead>
<tr>
<th>Cross</th>
<th>F&lt;sub&gt;1&lt;/sub&gt;</th>
<th>F&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Segregation ratios</th>
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<tr>
<td></td>
<td>Phenotype</td>
<td>Phenotypes</td>
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<tr>
<td><em>flp</em> x WT*</td>
<td>WT (32)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>WT:<em>flp</em></td>
<td>3.1:1&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td></td>
<td></td>
<td>(235:75)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><em>tmm</em> x WT</td>
<td>WT (6)</td>
<td>WT:<em>tmm</em></td>
<td>2.9 : 1&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td></td>
<td></td>
<td>(211:73)</td>
</tr>
<tr>
<td><em>flp</em> x <em>tmm</em></td>
<td>WT (21)</td>
<td>WT:*flp:*tmm:*dm&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.5:3.3:3.5:1&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>(384:147:157:45)</td>
</tr>
</tbody>
</table>

*Wild type.*

*Number of plants.*

*Double mutant.*

*χ<sup>2</sup>* test shows empirical ratio conforms to the expected ratio of 3:1 (*P* > 0.75).

*χ<sup>2</sup>* test shows empirical ratio conforms to the expected ratio of 9:3:3:1 (*P* > 0.10).
Figure 1. Possible pathway of stomatal development in *Arabidopsis*.

Stomatal precursor cell (meristemoid) formation and activity as described for various genera in the Brassicaceae. Sequence is redrawn from Landré (1972) by Fred Sack and is based upon 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 daughter cell becomes a non-stomatal epidermal cell (EC), and the smaller daughter cell becomes a meristemoid (M).

(B) and (C) The meristemoid then undergoes one to several asymmetric divisions. After each division, the larger daughter cell 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 GMC then undergoes a terminal symmetric division that produces two guard cells (GCs; stomatal pore not yet formed in D).
Figure 1.
Figure 2. Light microscopy of wild type and of stomatal mutants in *Arabidopsis*.

The abaxial epidermis of cotyledons is shown. Extra adjacent stomata are present in all three mutant phenotypes. Fresh epidermal peels stained with crystal violet. Darkly staining epidermal cells retained protoplasts and thus were stained after peeling. Bar = 50 μm for all micrographs.

(A) Wild-type (WT). Stomata are single and separated from each other by at least one epidermal cell. Arrowhead = meristemoid.

(B) *four lips* (flp). Abnormal stomatal units with paired stomata, some of which are laterally arranged (arrows). Arrowheads = meristemoids.

(C) *too many mouths* (tmm). Note clusters of stomata arranged in various angles, but not laterally.

(D) Double mutant (dm). Note the clusters are more compact compared to those of tmm. Some stomata in clusters are laterally arranged (arrows).
Figure 2.
Figure 3. Light microscopy of wild type and stomatal mutants.

The abaxial epidermis of cotyledons is shown. Whole mounts were cleared in ethanol and viewed with differential interference contrast optics. Bar = 40 μm for all micrographs.

(A) Wild type (WT).

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

(C) *tmm*.

(D) Double mutant (dm). Stomatal clusters include some stomata that are laterally arranged (arrowheads).
Figure 4. Meristemoids and stomata in developing wild-type cotyledons.

Light micrographs of the abaxial epidermis of 4-day-old cotyledons. Hand sectioned fresh tissue (A) or whole mount of the cotyledons (B-D) were viewed with differential interference contrast optics. Bar = 20 μm for all micrographs.

(A) Two meristemoids (arrows).

(B) A possible guard mother cell (arrow) appears rounder than the meristemoid at bottom center or the meristemoids in (A).

(C) A developing stoma (arrow) that has completed cytokinesis but the pore has not yet formed. This stoma is surrounded by four epidermal cells instead of three.

(D) Three stomata (arrows) in a developmental sequence with the youngest stoma at the left and the oldest at the right. Note stages of pore formation.
Figure 4.
Figure 5. Frequencies of stomatal units (SUs) with even or odd numbers of guard cells.

Data are from the abaxial epidermis of cotyledons in Experiment 2 (Table 1). Note the logarithmic scale on the ordinate. Standard Error bars are shown.

(A) All four genotypes (WT, flp, tmm, and 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 5.
Figure 6. Stomatal clusters

Epidermal peel (A) or a hand section (B) of the abaxial epidermis of unfixed cotyledons, viewed with differential interference contrast optics. Bars = 20 μm.

(A) tmm. This 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 the cluster. Large clusters with an odd number of guard cells are a unique feature of the phenotype of the double mutant.
Figure 6.
Figure 7. Large stomatal clusters in *tmm*.

Abaxial epidermis of whole mount of a *tmm* cotyledon from a 22-day-old seedling which was cleared in ethanol and viewed with differential interference contrast optics. The largest cluster contains stomata of different sizes, and some stomata appear to originate from different cell lineages. Also some stomata are not fully differentiated (arrows). Bar = 30 μm.
Figure 7.
Figure 8. Meristemoids and stomata in developing *tmm* cotyledons.

Light micrographs of the abaxial epidermis of 4-day-old cotyledons. Fresh tissue was sectioned by hand and viewed with differential interference contrast optics. Bar = 20 μm for both micrographs.

(A) Shows meristemoids (arrows), some of which are located adjacent to developing stomata (asterisks). Note the higher density of meristemoids in *tmm* compared to the wild type (Fig. 4A).

(B) Shows a region containing developing stomata, some of which are clustered.
Figure 9. Light microscopy of abnormal stomatal units in *flp* and the double mutant.

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). Bar = 10 μm for all micrographs.

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

(E) The double mutant. A rare SU is shown that has one normal and two "half" (truncated) guard cells all surrounding one stomatal pore (arrowheads).
Figure 9.
Figure 10. Abnormal divisions in committed meristemoids in developing *flp* cotyledons.

The abaxial epidermis of whole mounts of 4-day-old *flp* cotyledons viewed using differential interference contrast optics. Multiple guard cells or guard-cell-like cells appear to be produced from the same meristemoid or guard mother cell. The first division of the meristemoids (committed to producing guard cells) is probably symmetrical (central arrows) and produces roughly equal-sized daughter cells. The subsequent divisions of the two daughter cells can be either symmetrical (arrows) or asymmetrical (arrowheads). Bar = 20 μm for all micrographs.

(A) The committed meristemoid appears to have undergone two rounds of symmetrical divisions and is forming four guard cells.

(B) The committed meristemoid might have first divided symmetrically (arrows), with the resulting two daughter cells then dividing asymmetrically (arrowheads) to give rise to four guard cells or guard-cell-like cells.

(C) The committed meristemoid might have first divided symmetrically (arrows), and then one of the daughter cells divided asymmetrically (arrowheads) to produce three guard cells or guard-cell-like cells.

(D) A pair of adjoining, developing stomata appear to have been produced by two rounds of symmetrical divisions (arrows).
Figure 11. Diagram of locations of \textit{tmm} and \textit{flp} on chromosome 1.

The data were obtained by gene mapping with CAPS markers. The map positions of markers PVV4, NCC1 and ADH are shown in centimorgans in parentheses, and the position of marker PAB5 is between RFLP markers g4026 (85.5) and m315 (92.6) (\textit{Arabidopsis} Research Companion, Massachusetts General Hospital/Harvard). The genetic distance between \textit{tmm} and marker ADH is about 8.6 cM (6 recombinant chromosomes out of a total of 70 chromosomes from F\textsubscript{2} plants or from equivalent F\textsubscript{3} families with a \textit{tmm} phenotype). The distance between \textit{tmm} and PAB5 is approximately 18.1 cM (13 recombinant chromosomes out of 72 chromosomes). \textit{flp} is approximately 25.8 cM from PVV4 (17 recombinant chromosomes out of 66 chromosomes) and 3.0 cM from NCC1 (2 recombinant chromosomes out of 66 chromosomes). As controls, \textit{tmm} was found to be unlinked to NCC1, and \textit{flp} was unlinked to ADH. The genetic distances between PVV4 and NCC1, and between PAB5 and ADH determined by this study conform to previous data (\textit{Arabidopsis} Research Companion).
Figure 11.
Figure 12. Mean number of guard cells per SU or cluster by genotype.

Data are from Experiments 2 and 3 (see Table 1), which used 50 μmol m$^{-2}$ s$^{-1}$ and 100 μmol m$^{-2}$ s$^{-1}$ light intensities, respectively. All values between genotypes within (A) and (B) for each experiment are different from each other (t-test, $P < 0.05$). GCs = guard cells, WT = wild type, dm = double mutant.

(A) All SUs included in calculating the means.

(B) Only clustered SUs were used.
Figure 12.

No. of GCs per cluster

No. of GCs per SU

A

B
Figure 13. Abnormal meristemoids or guard mother cells in developing double mutant cotyledons.

Hand sections of unfixed 4-day-old cotyledons of the double mutant viewed using differential interference contrast optics. Some meristemoids or guard mother cells are abnormally round and large and appear to produce a guard-cell-like cell by an asymmetrical division. Bar = 20 μm for all micrographs.

(A) Shows abnormally rounded meristemoids or guard mother cells (arrows). The one in the center has divided asymmetrically (arrowheads).

(B) Two abnormal meristemoids or guard mother cells appear to have been adjacent to each other and to have divided asymmetrically to form one guard-cell-like cell and one larger cell (upper left, arrowheads). Note the presence of a similar asymmetrical division bottom left (arrowheads) and of two other meristemoids or guard mother cells (arrows).

(C) A similar asymmetrical division by an abnormal meristemoid or guard mother cell that may be at an earlier stage than in (A) and (B) (arrowheads). Note the presence of rounded meristemoids or guard mother cells (arrows).

(D) Two abnormal meristemoids or guard mother cells appear to be adjacent to each other (arrows).
Figure 14. Absolute numbers and densities of stomata, SUs, and ECs.

Data from Experiment 2 (see Table 1). The abaxial epidermis of 12 to 20-day-old cotyledons was examined. Statistical analysis ($t$-test at 0.05 level) indicates: (1) all three mutant genotypes had more stomata than did the wild type, (2) both $tmm$ and the double mutant had more SUs on either an absolute or a density basis than did the wild type, but only the absolute number of SUs in $fpl$ was greater than that of the wild type, (3) both the absolute number and the density of ECs decreased in the double mutant compared to the wild type, (4) the absolute number, but not the density, of ECs in $fpl$ was greater than that in the wild type.
Figure 14.
Figure 15. Absolute numbers and densities of stomata, SUs, and ECs.

Data from Experiment 3 (see Table 1). This experiment was performed as in Fig. 14. Statistical analysis (t-test at 0.05 level) indicates: (1) all three mutant genotypes had more stomata than did the wild type, (2) both \textit{tmm} and the double mutant had more SUs on either an absolute or a density basis than did the wild type, (3) the absolute number, but not the density of ECs in both \textit{tmm} and the double mutant was lower compared to the wild type.
Figure 15.
Figure 16. Arrested meristemoids and guard mother cells in the wild type and *fjp*.

Data from the abaxial epidermis of 22-day-old cotyledons. The absolute number (left ordinate) and density (right ordinate) of SUs in *fjp* are not different from those in the wild type, but there are more arrested meristemoids and arrested guard mother cells in *fjp* than in the wild type. Bars = Standard Errors.
Figure 16.
Figure 17. The ratio of ECs to stomatal units.

Data from Experiments 2-3 (see Table 1). Statistical analysis (t-test at 0.05 level) indicates that the means between *tmn*, the double mutant and the wild type are different for each data set, and the double mutant has the fewest ECs per SU among the three genotypes, but the numbers of ECs per SU in *fip* and the wild type are not significantly different. Bars = Standard Errors.
Figure 17.

WT  flp  tmm  dm

50 \mu mol m^{-2} s^{-1} light intensity

100 \mu mol m^{-2} s^{-1} light intensity
CHAPTER II

AN ARABIDOPSIS MUTANT DEFECTIVE IN CYTOKINESIS HAS LARGER CELL TYPES AND ABNORMAL ORGANOGENESIS

Cytokinesis, the process of cytoplasmic separation that usually follows karyokinesis (nuclear division), is one of the most prominent characteristics of all cellular organisms. The result of this cell division process is the birth of two daughter cells from a previous mother cell. Cytokinesis in plants normally results from the formation of a cell plate in the equatorial plane of the phragmoplast, an assemblage of microtubules. The young cell plate forms a disc that then extends laterally and fuses with the mother cell membrane (Esau, 1977; Gunning, 1982; Hepler, 1982). The leading edge of the cell plate is associated with a phragmoplast microtubule domain that becomes concentrically displaced during the centrifugal expansion of the plate.

Recently, Samuels et al. (1995) were able to detect more intermediate steps in cell plate formation based on studies on cryofixed tobacco cells. They proposed that cell plate formation consists of five stages: (1) the arrival of Golgi-derived vesicles in the equatorial plane, (2) the formation of thin tubes that grow out of individual vesicles and then fuse with each other to give rise to a continuous, interwoven, tubulo-vesicular network, (3) the consolidation of the tubulo-vesicular network into an interwoven smooth tubular network.
rich in callose, and then into a fenestrated plate-like structure, (4) the formation of hundreds of finger-like projections at the margins of the cell plate that fuse with the parent cell membrane, and (5) cell plate maturation that includes closing of the plate fenestrae and cellulose synthesis.

Such morphological studies provide a foundation for the genetic analysis of cytokinesis in plant cells, but only a few genes involved in cell plate formation or stabilization have been identified by mutational analysis (McCoy and Smith, 1983; Kitada et al., 1983; Liu, et al., 1995; Lukowitz, et al., 1996), and only one such gene, KNOLLE in Arabidopsis, has been cloned (Lukowitz, et al., 1996). Sequence analysis indicates that KNOLLE is a syntaxin-related gene product, which might be involved in vesicle transport. Thus, the mechanisms controlling plant cell cytokinesis are still largely unknown.

In contrast, many mutations affecting cytokinesis have been reported in eukaryotic microorganisms such as protozoa (Dee, et al., 1981; Sugai, 1984; Waddell, et al., 1987; Bailey et al., 1992), fungi (Chang and Nurse, 1993; DiDomenico, et al., 1994; Fankhauser and Simanis, 1994; Garcia-Bustos, et al., 1994; Harris, et al., 1994; Samejima and Yanagita, 1994), and animals (Hori, 1981; Gatti and Baker, 1989; Warn, 1990; Greenstein et al., 1994; Neufeld and Robin, 1994). Although some of these mutant phenotypes result from the completion of cytokinesis without karyokinesis (Samejima and Yanagita, 1994), most of these mutants are primarily deficient in cytokinesis. Molecular studies in these systems have revealed various gene products that are necessary for completion of cytokinesis, including cytoskeletal components, kinases, and homeo-domain proteins.
At a cellular level, cytokinesis defective mutants are characterized by the presence of multiple nuclei in one cell (Dee, et al., 1981; Kitada et al., 1983; Sugai, 1984; Waddell, et al., 1987; Gatti and Baker, 1989; Warn, 1990; Chang and Nurse, 1993; DiDomenico, et al., 1994; Fankhauser and Simanis, 1994; Garcia-Bustos, et al., 1994; Greenstein et al., 1994; Harris, et al., 1994; Neufeld and Robin, 1994; Liu et al., 1995; Lukowitz, et al., 1996). Alternatively, individual cells may have one large polyploid nucleus that is presumably formed by the fusion of multiple nuclei produced by karyokinesis (Hori, 1981; McCoy and Smith, 1983; Bailey et al., 1992, Liu et al., 1995; Lukowitz et al., 1996). Because cell size is usually positively correlated with nuclear DNA content, including in Arabidopsis (Melaragno et al., 1993), fewer and larger cells are another consistent feature of cytokinesis deficient mutants in multicellular organisms (McCoy and Smith, 1983; Kitada et al., 1983; Gatti and Baker, 1989; Warn, 1990; Greenstein et al., 1994; Neufeld and Robin, 1994; Liu et al., 1995; Lukowitz, et al., 1996). Also, incomplete cell wall protrusions have been observed in two of the cytokinesis defective mutants that have been reported for plants (Liu et al., 1995; Lukowitz, et al., 1996).

The exogenous application of inhibitors of cytokinesis such as caffeine, can mimic the phenotype of a cytokinesis mutant (Liu et al., 1995). It has also been shown that the ventral wall of stomata in plants treated with colchicine or caffeine, is often completely or partially absent, and these abnormal stomata lack pore formation (Reese, 1950; Galatis and Apostolakos, 1991; Terryn, et al., 1993). Such stomata should be another feature found in plant cytokinesis defective mutants.
The role of cytokinesis in organ development is a central question of developmental biology. But because cytokinesis defects are often lethal, and many conditional cytokinesis mutants are in unicellular or lower organisms, knowledge concerning this question in plants is minimal. Plants often exhibit a higher tolerance of various defects and show plasticity in development compared to other organisms, features which might be advantageous for studying this relationship.

This Chapter describes a recessive mutant of *Arabidopsis, cytokinesis defective* (*cyd*), which was found in a microscopy-based screen for stomatal mutants (Yang and Sack, 1995), but which has disrupted cytokinesis in several cell types. *cyd* also shows various abnormalities in vegetative and reproductive organs, and in reproductive processes including gametophytic development and embryogenesis.
MATERIALS AND METHODS

Plant Material and Culture

Plant material and growing conditions are the same as described in Chapter I. Plants were grown both in pots and on agar medium and were normally maintained under about 50 μmol m\(^{-2}\) s\(^{-1}\) (photosynthetic active radiation) at about 22°C.

Mutant Screening and Genetic Analysis

This cyd mutant was isolated using the same screening procedures described in Chapter I. Genetic crosses were performed as described in Chapter I. cyd was backcrossed to its wild-type (gll) Columbia parent two times, and then to the wild-type (GLI) Columbia line in the third backcross.

Quantitative Characterization of Phenotypes

The numbers and densities of stomata and non-stomatal epidermal cells (ECs) in mature cotyledons were determined as described in Chapter I. Stomata, meristemoids and guard mother cells were counted in three and six day old cotyledons as previously described, except that each microscopic field was 1.76 × 10\(^4\) μm\(^2\) for the young cotyledons.
(3 and 6 days old) instead of $3 \times 10^4 \, \mu m^2$ for fully expanded cotyledons.

Guard cell (ventral wall) length and width, and stomatal pore length were measured from the abaxial epidermis of cotyledons of 18-21 days old agar-grown plants, using an ocular reticle with the smallest unit of 3.5 \( \mu m \) and a total magnification of 400X.

For analysis of pollen size, pollen was collected from newly opened flowers by dipping the flowers in a drop of water on a microscopic slide. The polar and equatorial diameters of 20-54 pollen grains were measured for each flower within 10-20 minutes after pollen wetting. Promptness in data collection is required since the dimensions change as the pollen hydrates. Overall pollen size was determined as the product of the polar diameter and the equatorial diameter, a method which has been used for quantifying ploidy levels in \textit{Arabidopsis} (Altmann et al., 1994). 60 wild-type and 120 \textit{cyd} pollen grains from three flowers (one flower per plant for each genotype) were sampled.

To detect abnormalities in vegetative organ development, \textit{cyd} plants were grown under different conditions, i.e., (1) 30 \( \mu mol \, m^2 \, s^{-1} \) light intensity and 22\( ^\circ \)C, (2) 100 \( \mu mol \, m^2 \, s^{-1} \) and 22\( ^\circ \)C, (3) 30 \( \mu mol \, m^2 \, s^{-1} \) and 30\( ^\circ \)C, and (4) 100 \( \mu mol \, m^2 \, s^{-1} \) and 30\( ^\circ \)C. The abnormalities were assigned to four classes (see Results section). The phenotypes were determined using a dissecting microscope for 108-122 seedlings for each of the four growing conditions.

Class 3 and 4 plants were transferred to soil for the subsequent examination of floral development. These plants were maintained at their original temperatures (22\( ^\circ \)C or 30\( ^\circ \)C). The light intensity was about 50 \( \mu mol \, m^2 \, s^{-1} \) near the top of the pots, but due to stem elongation, light intensity varied along the inflorescence. A total of 75-99 flowers
from 20-22 plants for each of the four different growing conditions was examined under a dissecting microscope.

The quantitative differences that are reported were statistically significant at the 0.05 level ($t$ test).

**Mesophyll Cell Separation**

To determine nuclear number in individual cells, mesophyll cells were separated according to Pyke and Leech (1991). Briefly, pieces of wild-type and *cyd* rosette leaves were fixed in 3.5% (v/v) glutaraldehyde for 1 hour in the dark, and then placed in 0.1 M Na$_2$EDTA (pH 9) and shaken at 60°C for 2.5 hours to soften the tissue. Samples were stored at 4°C prior to fluorescent staining. After staining and mounting on a microscope slide, the coverslip was gently pressed to separate the mesophyll cells.

**Microscopy**

Micrographs of vegetative and reproductive organs were taken using a Zeiss dissecting microscope and a M35 camera system.

The techniques for bright-field and differential interference contrast microscopy were described in Chapter I.

For fluorescence microscopy, whole cotyledons were stained in 10 µg ml$^{-1}$ 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Melaragno et al., 1993). Macerated leaf samples for isolation of mesophyll cells were also stained simultaneously with 100 µg ml$^{-1}$ Calcofluor solution which stains cell walls (Hughes and McCully, 1975). Both dye
solutions were prepared as concentrated (100X) stock solutions that were diluted just before use. The samples were usually stained for one minute, and then were rinsed three times in distilled water and mounted for observation. The filter set used for fluorescence included a UG1 exciter filter (Fish Schurman), an LP420 barrier filter (Zeiss) and an FT 395 dichroic beam splitter (Zeiss). Micrographs were taken using a 35 mm camera attached to a Zeiss IM 35 microscope with a Leitz 100X NPL Fluotar objective using Kodak Vericolor 160 film.
RESULTS

Cytokinesis Is Defective in the cyd Cotyledon Epidermis

The mutant cyd was found in a screen for morphological stomatal mutants, and was identified by the presence of abnormal stomata that were larger or that had incomplete cytokinesis. Thus, characterization of the abnormal cellular phenotype focuses on the cotyledon epidermis because it is readily accessible and this allows reliable and rapid quantification.

The gl1 cotyledon epidermis contains two cell types, guard cells and other ECs. Guard cells and many of the ECs develop from meristemoids. After producing some ECs, meristemoids convert to guard mother cells that are the direct precursors of stomata. Some of the ECs can also divide to form more ECs and secondary meristemoids (see Chapter I). Thus, cell divisions are crucial for the formation of the epidermis.

Whole mounts of cyd cotyledons were examined for abnormal cell divisions in the abaxial epidermis. Incomplete cell wall formation was observed in both ECs (Figs. 18A-B) and stomata (Figs. 18C-H). On average, more than three ECs per cotyledon contained cell wall protrusions (abaxial epidermis sampled from 18 cotyledons). The cell wall protrusions extend either from one side (Figs. 18A-B, 18F and 18H) or more or less equally from both sides of the cell (Figs. 18C-D and 18G). The latter are usually shorter.
than wall protrusions found on only one side. In abnormal stomata these incomplete walls appear to be located where the ventral wall would normally form, since they bisect the oval-shaped stomata. Apparently because of incomplete cell wall formation, many stomata either do not develop pores (Figs. 18E-F), or they develop pores connected to only one protrusion (Fig. 18H). The differences in the extent of incomplete cell wall formation may reflect varying degrees of expressivity of the mutation. The presence in cty of normal stomata with pores and complete ventral walls is consistent with this notion (Figs. 18A and 18E).

Figs. 18C-D show a relatively rare stomatal abnormality where an unpaired guard cell adjoins a stoma. Cytokinesis was normal for the stoma itself, but incomplete for the adjacent guard cell. This could indicate that the earlier division (last asymmetrical meristemoid division) was impaired, but that the later division of the guard mother cell was normal.

To facilitate the description of the abnormal stomatal phenotype, four types of stomata are defined and classified as follows:
Type 1 - normal pore and ventral wall (regardless of cell size).

Type 2 - no pore or ventral wall.

Type 3 - one or two wall protrusions, but no pore.

Type 4 - pore attached to one wall protrusion with or without a second protrusion on the other side.

Figure 19. Stomatal types in *cyd* plants.

Most abnormal stomata without pore formation or normal cytokinesis can still be recognized as stomata since they have the overall shape and chloroplasts characteristic of mature stomata (Figs. 20A-B). These stomata also contain a wall swelling (Fig. 20C) that is an additional marker for their stomatal identity. This swelling appears to project from the outer cell wall, and it is usually elliptical in a paradermal plane, and is located at the mid-length where the stomatal pore would normally develop.

The percentages of the four types of stomata in the abaxial epidermis of the cotyledon are shown in Fig. 21. Type 1 stomata lack any cytokinesis defect, although they
are larger than those of the wild type on average (see following data) and comprised of 63% of all stomata sampled. Types 2-4 stomata were 21%, 4%, and 12% of all stomata, respectively.

In *cyd*, incomplete cytokinesis in ECs was also seen in the epidermis of organs other than cotyledons including rosette leaves, cauline leaves and sepals. Types 2-4 stomata were consistently seen in all these organs. Abnormal stomata and ECs with incomplete cytokinesis were never seen in wild-type plants.

Together, these data indicate that the *cyd* mutation generally results in incomplete cytokinesis in the *Arabidopsis* epidermis in several cell types and organs.

**cyd Stomata Are Bigger**

To compare the size of guard cells between wild type and *cyd*, guard cell length and width, and stomatal pore length were measured for stomata of normal morphology (Type 1) in the abaxial epidermis of cotyledons.

The average length of wild-type guard cells is 27.7 ± 0.3 μm (± Standard Error) compared to 40.7 ± 0.6 μm in *cyd*. Wild-type guard cells are 6.9 ± 0.04 μm wide versus 8.0 ± 0.1 μm in *cyd*. Stomatal pores are 18.0 ± 0.3 μm long in the wild type versus 28.3 ± 0.6 μm in *cyd*. Therefore, *cyd* stomata are much larger than wild-type stomata by all three parameters. Wild-type guard cells are 68% of the length of the *cyd* guard cells on average, while wild-type stomatal pores are 64% as large as *cyd* stomatal pores, indicating a proportional increase in both stomatal pore and guard cell length in *cyd*. But the average width of the guard cells increases much less than the lengths of guard cells and
stomatal pores in cyd, making the mutant stomata look relatively slender.

The histogram distributions of guard cells of different dimensions in the wild type and cyd are shown in Figs. 22 to 24. These figures clearly demonstrate that cyd stomata have a much larger range of sizes than the wild type.

**The Epidermis of cyd Cotyledons Has Fewer Cells**

Since the defects in cytokinesis would be expected to affect cell number, the numbers of cells of different types were determined in developing cotyledons (three and six day old agar-grown plants) and in fully expanded cotyledons (eighteen day old pot-grown plants). Three- and six-day-old cyd cotyledons had many fewer meristemoids/guard mother cells and stomata (total of normal and abnormal) than the wild type (Fig. 25). Within each genotype, most of the meristemoids and guard mother cells scored on day three later developed into stomata, because the total number of stomata (both normal and abnormal for cyd) at day six equals the sum of meristemoids, guard mother cells and stomata at day three (Fig. 25). These data suggest that the difference in number of stomata in cotyledons between the two genotypes was established before day three. In contrast, the rate of meristemoid/guard mother cell formation was comparable between cyd and the wild type from days four to six.

The numbers and densities of stomata (both normal and abnormal) and of ECs were determined for the abaxial epidermis in fully expanded cotyledons (Fig. 26). There is a consistent reduction in both stomata and ECs whether measured by absolute number or density. The average size of ECs is larger in cyd than in the wild type since the density of
cyd ECs is lower. The presence of larger and fewer ECs in cyd is consistent with the
observation that this mutation causes incomplete cytokinesis in some ECs (Figs. 18A-D).

The ratio of ECs to stomata in wild-type plants should reflect the frequency of cell
production by the meristemoids as well as by protodermal cells. This ratio was calculated
from the data set shown in Fig. 26. The values of ECs/stomata in the wild type and in cyd
are 2.6 ± 0.1 and 3.6 ± 0.2, respectively. Thus, more ECs are produced relative to
stomata in cyd.

Trichomes in cyd Have More Branches

The cyd mutation was also crossed into a GL1 background so that effects on
trichomes could be assessed. Results from the adaxial epidermis of the first pair of rosette
leaves show that 65% of cyd trichomes have four branches, 26% three branches, 8% five
branches, and 1% two branches. 96% of wild type trichomes have three branches, 3%
four branches, 1% two branches, and no trichomes have five branches (Fig. 27).

Multiple Nuclei and Nuclear Fusion Occur in cyd

As mentioned, a general characteristic of cytokinesis mutants is the presence of
more than one nucleus in one cell. The multiple nuclei can also fuse resulting in one
larger, polyploid nucleus.

The effect of the cyd mutation on nuclear number and morphology was studied by
fluorescence microscopy using DAPI staining of the abaxial epidermis of cotyledons (n =
15) and of macerated (isolated) mesophyll cells.
Cells in the wild-type epidermis of *Arabidopsis* have been shown to have a single nucleus, although the nuclei of cells of different sizes have different amounts of DNA (Melaragno et al., 1993). In fact, cells in all locations in *Arabidopsis* plants have varying DNA levels (Galbraith, et al., 1991). My own observations of wild-type tissues agrees with the above results, i.e., each epidermal cell always has one nucleus, and the larger the cell, the larger the nucleus. Therefore, only the *cyd* phenotype is described below.

In every *cyd* cotyledon surveyed, the majority of the ECs lacked cell wall protrusions and contained only one nucleus per cell. But in each cotyledon, there typically were a few ECs without wall protrusions that contained two or three nuclei per cell (Figs. 28A-G). In some cases, these nuclei were very close or physically in contact with each other (Figs. 28A-B and 28E-F). In other cases, the two nuclei were more separated (Figs. 28C-D). The nuclei in the same cell were not always the same size; note that one nucleus in Fig. 28E appears larger than the other two. Fig. 28G shows an EC with one nucleus with an extension; this nucleus is larger than any of the binuclei or trinuclei in other cells (Figs. 28A, 28C and 28E). This tail-like nuclear extension was repeatedly seen in *cyd* ECs.

In *cyd* ECs with cell wall protrusions, one large nucleus or two nuclei per cell were also observed (Figs. 29A-F). Nuclear size and morphology varied. The single nucleus in the EC shown in Fig. 29A is also larger than the nuclei present in cells with two nuclei (Figs. 29C and 29F). The two nuclei in Fig. 29C were connected by extensions that could represent a stage of nuclear fusion. The two nuclei in Fig. 28E were separated.
Nuclear phenotypes similar to those found in ECs were also found in stomata. Type 1 stomata always contained one nucleus per guard cell. But Type 2 stomata contained one or two nuclei per cell (Figs. 30A-B and 30C-F). The single nucleus in the Type 2 stoma shown in Fig. 29A is much larger than the nuclei present in cells with two nuclei (Figs. 30C and 30E). The two nuclei in Fig. 30C are separated, while the two nuclei in Fig. 30E appear to be in contact with each other. Similar nuclear phenotypes were observed in Type 3 stomata. In some cases, a single but larger nucleus was present in one cell (Figs. 31A-B). In other cases, two nuclei were present in single cells, and the nuclei were either connected (Figs. 31C-D), or separated (Figs. 31E-30F). Only two separated nuclei per cell were found in Type 4 stomata (Figs. 32A-B, and 32).

Nuclear number was also evaluated in mesophyll cells of cyd and in the wild type using DAPI and Calcofluor (to visualize cell walls) fluorescence. Most cyd, and all wild-type mesophyll cells appeared to be uninucleate, but in a few cases, two closely associated nuclei per cell were found in cyd mesophyll cells (Fig. 28H). Counting nuclei in mesophyll cells using conventional fluorescence microscopy is difficult because the chloroplasts usually block the view of the nucleus, and cell clumping often obscures the limits of individual cells.

To determine whether the extent of incomplete cytokinesis is related to the number of nuclei per cell, the number of nuclei in each of the three types of abnormal stomata was quantified (Fig. 33). Of the 70 Type 2 stomata scored, 40% were binucleate and 60% were uninucleate. Of the 30 Type 3 stomata scored, 77% were binucleate and 23% were uninucleate. All 25 Type 4 stomata that were sampled were binucleate. These data
indicate that the binucleate phenotype is positively correlated with the extent of cell separation, i.e., the greater the cell partitioning, the higher the percentage of the binucleate cells.

Of the 11 ECs with cell wall protrusions that were scored for this phenotype, 6 contained two nuclei, indicating that cell partitioning may also correlate with nuclear number in ECs.

Altogether, these data show that cells with absent or incomplete cytokinesis are likely to have multiple nuclei or single larger nuclei.

cyd Is a Partially Lethal Nuclear Recessive Mutation

cyd was backcrossed to wild-type plants (gl1 and GL1) for genetic analysis (Table 5). The F1 plants (n=17) from two independent crosses (with cyd as the pollen donor) all showed the wild-type cellular phenotype. This indicates that cyd is a recessive mutation. These F1 plants were selfed and the phenotypes of the F2 plants were quantified. The segregation ratio of wild-type to cyd plants in the F2 population is 4.4 : 1 instead of 3 : 1, suggesting that cyd is a partially lethal nuclear mutation. A similar segregation ratio was found in F2 populations when the wild type was the pollen donor in the backcross. So far, only one allele, cydl-1, has been found.

The seed set of cyd plants is very poor, which also suggests that cyd is partially lethal. In one experiment, cyd siliques had an average of only 11 relatively normal-looking seeds, compared to 34 in the wild type grown under comparable conditions (Table 6). In addition, cyd siliques contained some apparently underdeveloped and collapsed seeds,
whereas all wild-type seeds looked essentially normal.

**cyd Pollen Is Larger**

Both *cyd* and wild-type pollen grains are ellipsoidal, but they differ in size. The index of pollen size employed was the product of the polar and equatorial diameters (Altmann et al., 1994). Using this metric, *cyd* pollen was $754 \pm 15 \mu m^2$ on average which is larger than the wild type value of $608 \pm 7 \mu m^2$. *cyd* pollen also shows a much greater range of sizes than wild-type pollen (Fig. 34). About 64% of *cyd* pollen grains fell into the full range of wild-type pollen sizes.

**Female Gametophyte Production in *cyd* Is also Affected**

In three independent crosses of female *cyd* plants with male wild-type plants, the seed set of all the siliques (2-3 sampled per cross) was poor with usually no more than two developed seeds per silique. In contrast, when *cyd* was used as a pollen donor in six independent crosses with wild-type plants, seed set was much better, with usually 10 to 20 seeds per silique. The F1 plants of both sets of crosses also had poor seed set, but they otherwise had a wild-type phenotype. These data suggest that female gametophyte production in *cyd* is somewhat defective.

**Abnormalities in *cyd* Vegetative Development**

Because defects in organogenesis were seen in initial examinations of *cyd*, and because light and temperature might affect cell division, *cyd* vegetative development was
evaluated after plant culture at different light intensities and temperatures (30 μmol m⁻² s⁻¹ at 22°C or 30°C, or 100 μmol m⁻² s⁻¹ at 22°C or 30°C). Seeds were sown on supplemented agar medium with 2% sucrose, and 8-10 day old seedlings were observed directly in the culture plate using a dissecting microscope. Seedlings were scored for various abnormalities. After scoring, many seedlings were transplanted from agar to pot culture to evaluate their ability to develop into mature plants.

All cyd plants showed at least some vegetative abnormalities. The same range of abnormalities in vegetative development was observed in all four sets of cultural conditions. The abnormalities found in a sample of 447 plants (108-122 for each culture condition) were assigned to four classes as follows:

Class 1 seeds did not germinate. These constituted about 25% of all seeds.

Class 2 seedlings arrested in development after forming four or fewer abnormal leaves and an abnormal root. For example, some seedlings had only one cotyledon and no apparent root (Fig. 35C), or they had only limited root development (Fig. 35D). This class represented 19% of all plants.

Class 3 seedlings usually developed into mature, fertile plants following transfer from agar to pot culture. But they had an obvious, asymmetrical seedling morphology due to, e.g., a missing or reduced cotyledon (Fig. 35E) or a leaf (Fig. 35F), to fusion or lobing of cotyledons or leaves (Figs. 36A-C), or to the growth of extra deformed tissue (Figs. 36B and 36D). Sometimes, a spine-like structure with apparent anthocyanin accumulation formed in the position of a leaf (Fig. 35F). Tissue growths of unknown organ identity sometimes formed at the shoot apex, a growth never seen in the wild type (Fig. 36B).
Organ extensions connected to foliar organs in what appeared to be an adventitious shoot (Fig. 36D) were found twice in the survey. Overall, about 20% of all plants were assigned to this class.

Class 4 seedlings looked relatively normal and they developed into mature, fertile plants following transfer to pot culture. But even in these plants, the shape and the arrangement of leaves was irregular compared to those of the wild type. For example, the first pair of leaves were not the same size, and they had larger teeth than the wild type (Figs. 35A-B). This class comprised of about 36% of all plants.

Fig. 37 demonstrates that the same range of abnormalities was observed in seedlings grown under all four sets of cultural conditions. The greatest percentage of healthy mutant plants was obtained at 30 μmol m⁻² s⁻¹ and 22°C. But overall, the variations in temperature and light intensity did not have a dramatic impact on the development of the mutant phenotype.

Abnormalities in cyd Floral Development

To determine whether cyd affects floral development, seedlings grown on agar for 10 days were assigned to Classes 3 and 4 based upon vegetative features and then were transferred to pot culture (50 μmol m⁻² s⁻¹ light intensity), and maintained at their original temperature (22 ± 1 or 30 ± 2°C).

The wild-type Arabidopsis flower consists of four whorls: the outermost whorl has four sepals, the next whorl has four petals, then a whorl with six stamens, and the innermost whorl has two fused carpels (Bowman, 1994). cyd flowers can have a
morphology like the wild type or can exhibit various abnormalities. More than fourteen types of abnormalities were found and arranged in order of frequency of occurrence with 1 the most frequent and 14, a miscellaneous category, the least (see Fig. 38). The numbers in parentheses below refer to these types and their frequencies. These floral abnormalities fell into several broad categories:

**Fusions between organs in the same whorl.** Fusions were seen between: two (2) or three (9) sepals (Figs. 39A-B), two petals (12; Fig. 39C), and two (5), three (11), or four stamens (14; Figs. 39D-F). The extent of fusion varied from only at the base or along the length of the organs.

**Fusions between organs in adjacent whorls.** Varying amounts of fusion were seen between: a sepal and a petal (14; Fig. 40A), a petal and a stamen (4) containing pollen (Fig. 40B), a stamen and a carpel (3; Figs. 40C-D), and an anther and part of a petal, identified by the characteristic papillar cells of a normal petal (14; Fig. 40E).

**Missing or reduced parts.** *cycl* flowers were found to contain four normal-sized stamens (7), four normal-sized stamens plus one reduced stamen (14; Fig. 41A), five normal stamens (1; Fig. 41B), five normal stamens plus one reduced stamen (6; Fig. 41C), and reduced petals (10; Fig. 41D). Sometimes, a reduced stamen was associated with another small stamen-like structure as if the flower contained seven stamens (8; Fig. 41E). But the tissue associated with a reduced stamen could also be amorphous and unidentifiable (8; Fig. 41F).

**Other Abnormalities** include deformed sepals (14), petals (14), stamens (14), carpels (14), fusions between half of a split anther and its own filament (14; Fig. 39G)
These abnormalities were analyzed quantitatively. Of all flowers sampled (n = 354) at both temperatures, 55% were visibly abnormal with 1-4 abnormalities in each flower. The percentage of different types of floral abnormalities out of all abnormalities (n = 246) analyzed is summarized in Fig. 38.

The most frequent abnormalities included five stamens (28%) and two fused sepals (26%). The next most frequent abnormalities included fusions between a stamen and a carpel (9%), a stamen and a petal (9%), and between two stamens (8%).

Stamens were the organ that was most likely to show a decrease in organ number and a reduction in organ size. But the minimum number of stamens observed was four.

cyd plants grown at 22 ± 1°C and 30 ± 2°C showed the same range of floral abnormalities with respect to severity and type, indicating that this difference in temperature did not affect flower morphology. Both seedlings with Class 3 or 4 vegetative abnormalities had comparable percentages of abnormal flowers (Fig. 42). A higher light intensity (100 vs 30 μmol m⁻² s⁻¹) during the first 10 days of seedling growth might promote normal flower production at both 22°C and 30°C compared to the 30 μmol m⁻² s⁻¹ light intensity (Fig. 43).
DISCUSSION

*cyd* is a partially lethal, recessive mutation of *Arabidopsis* resulting in defective cytokinesis. Several cell types such as stomata, ECs, and mesophyll cells contain multiple nuclei in one cell. Also, incomplete cell walls are found in ECs and in some stomata, and other stomata lack dividing walls entirely. The cotyledon epidermis of *cyd* contains fewer but larger cells. Also consistent with a cytokinesis defect are various other abnormalities including larger nuclei in larger cells, larger stomata and pollen grains, more branches on trichomes, poor seed set, and missing, reduced, or fused vegetative and floral organs. Thus, the *CYD* gene product is probably required for normal cytokinesis in many cell types in several organs. This mutant provides a unique opportunity to analyze the relationships between cytokinesis defects and organ development in *Arabidopsis*.

**Cellular Phenotype Indicates Defective Cytokinesis**

As in cytokinesis defective mutants in other kingdoms, plant cytokinesis defective mutants are characterized by the presence of several nuclei in one cell, by higher DNA content in larger cells, by a reduction in cell number, and in some cases by the fusion of the multiple nuclei within a cell (Kitada, et al., 1983; McCoy and Smith, 1983; Liu, et al., 1995; Lukowitz et al., 1996). Some plant cytokinesis mutants also contain cell wall
protrusions, i.e., incomplete cell walls (Kitada, et al., 1983; Liu, et al., 1995; Lukowitz et al., 1996).

The cyd mutant in Arabidopsis described here shows all these characteristics of plant cytokinesis defective mutants. Single larger nuclei, single cells containing two or three nuclei, and cell wall protrusions were found in stomata and ECs in cotyledons, rosette leaves, cauline leaves and sepals of cyd plants. Binucleate mesophyll cells were also found in cyd leaves. The abaxial epidermis of a cyd cotyledon contains larger and fewer stomata and ECs than the wild type. These abnormalities seem directly or closely associated with a cytokinesis defect in these cells.

The occurrence of several nuclei in one cell, wall protrusions, fewer cells, and the absence of a dividing wall in abnormal stomata, indicates that karyokinesis is unlikely to be impaired in cyd, and that the presence of multiple nuclei in one cell probably results from the absence of cytokinesis. It is also unlikely that the multiple nuclei in one cell result simply from an overproduction of nuclei, since there usually are only two nuclei per cell and since a severe reduction in cell number was found in the abaxial epidermis of the cotyledon (Fig. 26). Presumably the single larger nuclei form through fusion of the multiple nuclei that otherwise would be separated by an intervening cell wall (Fig. 28). But DNA levels have not yet been measured in larger cyd nuclei in Arabidopsis.

Larger Cells Could Result from Endopolyploid Cell Lineages

Larger ECs (Melaragno, et al., 1993), stomata (data not shown), and pollen (Altmann et al., 1994), and trichomes with more branches (Martin Hülskamp, personal
communication) are all indicators of polyploidy in Arabidopsis. cyd shows all these phenotypes suggesting that they result from endopolyploid cell lineages. It is hypothesized here that specific cell lineages could become endopolyploid after nuclear fusion following a cytokinesis defect.

It is unlikely that cyd itself is just a polyploid line. In tetra- and hexaploid Arabidopsis, all stomata and pollen are larger, and no abnormal stomata with incomplete ventral walls were found (data not shown). But in cyd, almost half of stomata and pollen grains are the same size as in the wild type (Figs. 22-24, and 34). Furthermore, cyd is clearly a recessive mutation and its cellular phenotype consistently segregates in F₂ populations after three consecutive backcrosses. These data support the hypothesis that some cyd cells become endopolyploid de novo during development due to a leaky mutation.

Cell Differentiation and Cytokinesis Can Be at least Partially Uncoupled

Type 4 stomata (see Results) are obviously stomata since a pore is present, though the ventral wall is still incomplete (Fig. 18H). The overall shape of Type 4 stomata and its chloroplasts also closely resemble normal stomata.

Shape and chloroplast differentiation also allow Types 2 and 3 stomata to be identified as stomata even though they lack pore formation. Types 2 and 3 stomata can also be recognized as stomata because they have a swollen outer paradermal cell wall in the position of a stomatal pore although no pore is present (Fig. 20C).
Thus, stomatal differentiation can be partially uncoupled from cytokinesis in abnormal cyd stomata. These data also indicate that stomatal pore formation may require a critical length of a ventral wall since stomatal Types 2 and 3, which lack a ventral wall entirely or which contain only a short ventral wall, never form a pore. However, it is possible to form a pore without ventral wall extensions, since the pore in stomata of the moss Funaria is the only ventral wall present so that the stoma consists of one, binucleate guard cell (Sack and Paolillo, 1983).

Consistent with a partial uncoupling of cytokinesis and stomatal differentiation in cyd, mesophyll cells with two nuclei still are clearly recognizable as mesophyll cells based on their shape and chloroplast number and size (Fig. 28H). This indicates that although at least one cell cycle was blocked, they still differentiated into mesophyll cells.

Cytokinesis is an iterative process while cell differentiation is usually a progressive process. The two processes are normally coordinated during development in order to have the right number of cells following the right cell fate at the right time. But this coordination does not necessarily mean that the two processes are obligately coupled. These data for cyd suggest that cell differentiation can at least partially proceed without cytokinesis.

cyd Affects Cell Cycles Leading to Stomatal Formation

There are usually at least three classes of cell division events occurring during stomatal development: (1) the division of a protodermal cell to form a primary meristemoid, or the division of an EC to form a secondary meristemoid. (2) the
division(s) of the meristemoid to produce one or more ECs, and (3) the division of the guard mother cell (derived from the meristemoid) to form two guard cells.

At least two classes of these divisions are affected in cyd. cyd cotyledons have fewer meristemoids and stomata than the wild type (Figs. 25-26). Although it is not clear whether this reduction occurs in primary and/or secondary meristemoids, these data do indicate that the number of the first class of divisions leading to stomatal formation is reduced. The third class of divisions are also affected since there are many abnormal stomata with absent or incomplete ventral walls. Since there are fewer ECs in cyd cotyledons than in the wild type, there must be fewer total divisions in the second class, but it is not clear whether this is due to a reduction in meristemoid number and/or to fewer cell divisions of the meristemoids that are present.

Caffeine Mimics the cyd Abnormal Cellular Phenotype

Caffeine is a strong inhibitor of cell plate formation in dividing plant cells (Hepler and Bonsignore, 1990). Caffeine-treated seedlings of maize and Arabidopsis have abnormal stomata that resemble those of cyd, i.e., stomata lacking a ventral wall (Galatis and Apostolakos, 1991; Terryn et al., 1993). Also the stomatal wall swelling found in cyd occurs as well in caffeine-treated maize stomata with a cytokinesis defect (Galatis and Apostolakos, 1991). A similar stomatal phenotype was also seen in colchicine-treated Petroselinum crispum plants (Reese, 1950).

Abnormal stomata similar to those in cyd also occasionally occur in wild-type plants (Dehnel, 1961). These abnormal stomata might be caused by the occasional
malfunctioning of a homolog of \textit{CYD} in guard mother cells.

The effects of caffeine on cytokinesis have been studied in cell types in addition to stomata. Caffeine treatment produces incomplete cell walls in \textit{Tradescantia} stamen hair cells (Hepler and Bonsignore, 1990) and pea root cells (Liu et al., 1995). Hepler and Bonsignore also showed that early cell plate formation appears normal in the stamen hair cells but that caffeine inhibits the stabilization of the plate and somehow allows the dissolution of the cell plate/young cell wall. It will be interesting to determine whether this same stage of cytokinesis is also defective in \textit{cyd} plants.

Since cell plate formation starts at the cell center, it may be significant that in both cytokinesis mutants and in caffeine-treated plants, the incomplete walls are never present as an island in the cytoplasm, but instead are connected to the parent cell walls. This might imply that cell plates are stabler where they join the parent wall than in the cell center.

\textbf{The Extent of Cytokinetic Partitioning Correlates with Nuclear Number in Abnormal Stomata}

Stomatal Types 2-4 in \textit{cyd} have varying degrees of cytokinetic partitioning, with Type 2 stomata having the least partitioning (no ventral wall), Type 3 stomata being intermediate (partial ventral wall but no pore formation), and Type 4 stomata having the most (stomatal pore and partial ventral wall). Interestingly, the percentage of cells with one nucleus is the highest in Type 2 stomata, lower in Type 3 stomata, and virtually zero in Type 4 stomata; no Type 4 stomata had just one nucleus. These data indicate that
nuclear number in these abnormal stomata is positively correlated with the extent of cytokinetic partitioning.

This could result if the larger cell partitionings interfere with the fusion of the two nuclei produced by normal karyokinesis. Alternatively, both the extent of cell partitioning and the frequency of nuclear division might be coordinated. But based upon the presence of larger nuclei (Figs. 30A and 31B) and nuclei in contact (Figs. 30E and 31D) in Types 2 and 3 stomata, it is more likely that nuclear fusion regularly occurs. If so, the presence of an incomplete wall may protect against nuclear fusion.

Poor Seed Set in cyd Might Be Due to Defective Gametogenesis

Seed set in cyd is only about one-third that of the wild type (Table 6). Female gametophyte production is probably affected in cyd since seed set is much poorer when female cyd plants are crossed to male wild-type plants compared to reciprocal crosses. cyd plants also have much larger pollen grains than the wild type. Work with a postmeiotic cytokinesis mutant in alfalfa suggests that larger pollen is less capable of fertilization (McCoy and Smith, 1983). Thus, the lowered seed set in cyd may be caused by defects in the production of both female and male gametophytes, suggesting that cytokinesis may be defective in these processes.

Cytokinesis Defects and Organogenesis

Two plant mutants with cytokinesis defects, cyd in pea and knolle in Arabidopsis, are lethal (Liu et al., 1995; Lukowitz et al., 1996). In contrast, the Arabidopsis cyd
mutant still allows many plants to set seeds. Thus, this cyd mutant enables the assessment of the relationships between a cytokinesis defect and organogenesis.

Abnormalities in cyd vegetative and floral organ development show significant similarities. They both include missing and reduced organs, and fusions between adjacent organs. The missing organs could result from a lack of divisions in critical cells during the formation of organ primordia. Similarly, the reduced organs could result from reductions in cell divisions later in organogenesis, and fusion could result from the lack of divisions in cells essential for forming boundaries between adjacent organs. Thus, these defects in organogenesis may reflect the supracellular consequences of a cytokinesis defect.

Clearly, more research is needed to establish that these organ defects are a direct consequence of the cytokinesis defect, but cyd probably provides a unique opportunity to address these questions.

Floral Organ Fusions Relate to The Positions of Primordia in Whorls

It has been shown that during wild-type Arabidopsis flower development, the sepal primordia develop much earlier than other floral organ primordia. Also, stamen primordia develop close to petal and gynoecium primordia as well as to each other. Petal primordia alternate with stamen primordia in different whorls (Smyth, et al., 1990). The most frequently found fusions between floral organs in cyd reflect the developmental proximity of primordia. Thus, the most common cyd fusions are between two sepals, a petal and a stamen, a stamen and a carpel, and two stamens. If these fusions result from incomplete cytokinesis, then the separation of the above pairs of floral organs probably requires the
proper separation of critical, strategically placed cells to create a boundary between primordia.

Conversely, rare fusion patterns probably represent spatial and temporal distance between primordia such as between a petal and a sepal. Similarly, it is unlikely that two or more fusion events would occur simultaneously such as between a petal and two neighboring stamens.

Possible Explanations for the Range in Severity of cyd Phenotype

On the one hand, the cyd mutation is completely penetrant in the population because every cyd plant shows at least some aspects of the mutant phenotype. On the other hand, this cyd allele is leaky because a range of severity exists in the phenotypes of cyd plants, e.g., from ungerminated seeds to more or less normal-looking plants. This leakiness could be explained if the cytokinesis defect affected all cells randomly and if the consequences of a defect depended on the essentiality for development of the cell affected. This could explain why some cells seem unaffected. It could also explain the range of severity if essential cells were not always affected. Similar explanations were presented for the range of severity in the gnom mutation in Arabidopsis which randomizes the plane of cell division and the orientation of expansion (Mayer et al., 1993; Shevell, et al., 1994).

In any case, it is not clear why the cyd mutation does not affect all cell divisions. Possible explanations for this are that the existing allele is weak, or that other functional homolog(s) of CYD are present that can partially compensate for the loss of CYD function.
Once *cyd* seedlings produce more than four rosette leaves, they usually mature into adult plants. This would suggest that once the apical meristem is well established, there are enough initials for development even if some undergo defective cytokinesis. Alternatively, an established apical meristem might be less affected by the mutation. Liu et al. (1995) reported that in the pea *cyd* mutant, the apical meristem does appear to be unaffected, although this mutant has a more severe phenotype than the *Arabidopsis cyd*.

**cyd Is Unlikely to Be an Allele of the *knoile* Mutant**

Recently, an *Arabidopsis* gene *KNOLLE* has been cloned (Lukowitz et al., 1996). Sequence analysis of this gene product revealed that it is related to syntaxins shown to be involved in vesicle targeting in other organisms (Lukowitz et al., 1996). Presumed null mutations of *KNOLLE* cause cytokinesis defects in the embryo and early seedling lethality. But *KNOLLE* is not required for the normal development of the gametophytic generation since 25% of the seeds produced by heterozygous plants are mutants. In contrast, *cyd* is required for the normal development of the gametophytic generation, and it is only partially lethal. These phenotypic differences between *cyd* and *knoile* make it unlikely that *cyd* is a weak allele of *knoile*. However, a genetic cross between the two mutants and gene mapping of *cyd* (in progress) are needed to establish that they are indeed different genes.
Conclusions

An *Arabidopsis* mutant, *cyd*, has been identified, which shows defective cytokinesis in several types of cells in different organs. *cyd* has features generally found in other cytokinesis mutants such as multiple nuclei, or a single but larger nucleus in one cell, cell wall protrusions, larger cell types, and fewer cells. Since many *cyd* plants are fertile and produce seeds, this mutant provides the opportunity to evaluate the relationships between a cytokinesis defect and organ development. Missing or reduced organs and fusions between adjacent organs were consistently seen during both vegetative and floral organ development and presumably result from the failure to divide of essential cells in primordia.
**Table 5. Genetic Analysis of cyd**

<table>
<thead>
<tr>
<th>Cross</th>
<th>F₁ Phenotype (^a)</th>
<th>F₁ Phenotypes</th>
<th>F₂ Segregation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \sigma \text{cyd} \times \sigma \text{wild type} )</td>
<td>wild type</td>
<td>wild type : cyd</td>
<td>4.4 : 1(^b) (189 : 58)(^c)</td>
</tr>
</tbody>
</table>

\(^a\) At a cellular level, i.e., presence of incomplete cytokinesis.

\(^b\) \(\chi^2\) test shows empirical ratio does not conform to the expected ratio of 3 : 1 (\(P < 0.05\)).

\(^c\) Number of plants examined.
<table>
<thead>
<tr>
<th></th>
<th>Normal Seed Morphology</th>
<th>Collapsed Seeds</th>
<th>All seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (n = 21)*</td>
<td>34.0±0.8(^b)</td>
<td>0.3±0.1</td>
<td>34.3±0.8</td>
</tr>
<tr>
<td>cyd (n = 12)</td>
<td>11.2±1.1</td>
<td>4.0±0.6</td>
<td>15.2±1.2</td>
</tr>
</tbody>
</table>

* Number of siliques examined.

\(^b\) Mean ± Standard Error.
Abaxial epidermis of whole mounted 7-15 day-old cotyledons observed with differential interference optics. Partial and complete loss of cell partitioning was seen in both ECs and stomata. Bar = 20 µm for all micrographs.

(A) A cell wall protrusion (arrow) extends from one side of the parent wall in an EC in contact with a stoma of normal morphology. The anticlinal wall in the EC was incomplete in all planes of focus.

(B) Similar to (A) but in a larger EC (arrow).

(C) and (D) Show what appears to be a guard cell (on the right) adjoined to a stoma. Note the presence of two cell wall protrusions from two sides of the parent wall (arrows). The focus is closer to the surface in (C) and deeper in (D). This type of abnormality was seen only rarely.

(E) The cell in the center appears to be a stoma based upon chloroplast differentiation, overall shape and cell size. However, no ventral wall or pore is present. Two normal stomata are nearby.

(F) Similar to (E) but with a short cell wall protrusion (arrow).

(G) The abnormal stoma has two cell wall protrusions (arrows) that may be located where the ventral walls would normally form. Note the two nuclei that are connected in the center of the stoma (arrowheads).
(H) An abnormal stoma has a pore but lacks a ventral wall in one pole of the cell.

Note that the pore is located closer to one end of the cell.
Figure 20. Type 2 and 3 stomata contain normally differentiated chloroplasts.

Epidermal peels were lightly stained with 0.05% (w/v) crystal violet and viewed using differential interference contrast optics. Bar = 10 μm for all micrographs.

(A) Type 1 stomata have a normal morphology but are much larger than wild-type stomata.

(B) Type 2 stomata lack a ventral wall but contain chloroplasts that are morphologically indistinguishable from those in Type 1 stomata. Although not shown, Type 3 stomata also contain chloroplasts with the same morphology.

(C) Types 2 and 3 stomata consistently show an elliptical swelling in the center of the outer paradermal cell wall (shown here in a Type 2 stoma; arrow). This structure appears to be located where the pore would normally develop. Note that the focus is close to the surface of the cell.
Figure 21. Percentage of different types of stomata in *cyd* plants.

Data were collected from the abaxial epidermis of 18 day old cotyledons from pot-grown plants (20 cotyledons per genotype). Stomatal types are designated as in Fig. 19 in text. For morphology of these stomatal types, see also Figs. 18E-H.
Figure 22. Guard cell length in wild-type and cyd cotyledons.

Data were from 200 guard cells of 10 cotyledons (20 guard cells randomly selected from each cotyledon) for each genotype from 18-19 day old, agar-grown seedlings. Guard cell length refers to the straight, maximal length of the ventral wall. In cyd, only Type 1 stomata were measured. Note that cyd guard cells are usually much longer than those of the wild type.
Figure 22.

Graph showing the percent of all guard cells among different types.

- Wild type
- *cph4* mutant

Guard cell length (μm)

Percent of All Guard Cells (%)
Figure 23. Guard cell width in wild-type and *cyd* cotyledons.

Data were collected from the same guard cells used for the guard cell length measurements. The guard cell width was measured at the mid-depth of focus of the guard cell. *cyd* guard cells are generally wider than those of the wild type.
Figure 23.
Figure 24. Stomatal pore length in wild-type and cyd cotyledons.

Data were collected from the same stomata used for the guard cell length and width measurements. The stomatal pore length refers to the length of the actual opening of the pore along the long axis of the stoma. Note that cyd pores are usually longer than those of the wild type.
Figure 24.

Percent of All Stomata

Stomatal Pore Length (μm)

Wild type

chg

19
5
12
26
33
40
54
47
Figure 25. Numbers of stomata and stomatal precursors in young wild-type and cyd cotyledons.

The numbers of meristemoids/guard mother cells and stomata in the abaxial epidermis of three and six day old, agar-grown seedlings were determined as described in the "Quantitative studies of mutant cotyledon phenotypes" in Chapter I, except that each microscopic field represented 0.0176 mm\(^2\) in area. Because it is often difficult to distinguish guard mother cells from meristemoids, both cell types were scored together. Compared to the wild type, cyd has significantly fewer meristemoids/guard mother cells and stomata in the three day old cotyledons, and fewer stomata in the six day old cotyledons. Within each genotype, the number of stomata in six day old cotyledons essentially equaled the total of all meristemoids/guard mother cells and stomata in three day old cotyledons indicating that during the three day period between sampling, all the meristemoids and guard mother cells developed into stomata (and additional meristemoids/guard mother cells formed). Both the wild type and cyd had the same number of meristemoids/guard mother cells in six day old cotyledons.
Figure 25.
Figure 26. Numbers and densities of stomata and epidermal cells in mature wild-type and cya cotyledons.

The data were collected as described in Chapter I. The plants are the same ones used for the data in Fig. 20. The abaxial epidermis of mature cya cotyledons contains fewer stomata (including both normal and abnormal ones) and ECs than the wild type on both an absolute number and density basis. These data are comparable to young cotyledons (Fig. 25) for agar-grown plants, except that there are fewer stomata in both the wild type and cya in the pot-grown plants (this figure). Pot-grown plants typically have fewer stomata than agar-grown plants regardless of genotype.
Figure 26.
Figure 27. Numbers of trichome branches in the first leaf of wild type and cyd plants.

Branch numbers of all trichomes in the adaxial epidermis of the first leaf from both the wild type and cyd one month old plants (a total of 10 leaves from 10 plants for each genotype) were scored using a dissecting microscope. Most trichomes in cyd had four branches while the wild-type plants mostly contained three-branch trichomes.
Figure 27.
Figure 28. Nuclear numbers in *cyd* epidermal cells without cell wall protrusions and in mesophyll cells.

DAPI stained abaxial epidermis of 14 to 15-day-old whole mounted cotyledons from agar-grown plants were examined with fluorescence microscopy. DAPI and Calcofluor stained macerated mesophyll cells were also examined (H). (B), (D) and (F) are the bright-field images corresponding to (A), (C) and (E), respectively. Most ECs and mesophyll cells had only one nucleus, but two to three nuclei per cell were occasionally found in these two types of cells. The nuclei within a cell that contains several nuclei were never found to be as large as some single nuclei. Bar = 20 μm for all micrographs.

(A) and (B) Two close but distinct and equal-sized nuclei (arrows) in the same epidermal cell.

(C) and (D) Two separated and roughly equal-sized nuclei (arrows) are present in one epidermal cell.

(E) and (F) Three closely associated nuclei (arrows) - two equal-sized and a larger nucleus - are present in the same epidermal cell.

(G) One larger nucleus in an epidermal cell. It appears to have a nuclear extension (arrow).

(H) A mesophyll cell that appears to contain two closely associated nuclei (arrows).
Figure 28.
Figure 29. Nuclear number and morphology in *cyd* epidermal cells containing cell wall protrusions.

DAPI fluorescence. (B), (D) and (F) are the bright-field images corresponding to (A), (C) and (E), respectively. One or two nuclei per cell were found. Bar = 20 μm for all micrographs.

(A) and (B) A single, large nucleus (arrow) is present in an EC that has two cell wall protrusions (arrowheads).

(C) and (D) Two connected nuclei (arrows) in an EC with a cell wall protrusion (arrowhead). The two nuclei may be in the process of fusion and each has two extensions.

(E) and (F) Two separated nuclei (arrows) in an EC with a cell wall protrusion (arrowhead).
Figure 30. Nuclear number in *cyd* Type 2 stomata.

DAPI fluorescence. (B), (D), and (F) and are the bright-field images corresponding to (A), (C), and (E), respectively. One or two nuclei per cell were found. Nuclear size varies with single nuclei tending to be larger. The cells can be recognized as abnormal stomata because of their overall shape and size, and the characteristic structure in the cell wall where a stomatal pore would have normally formed (Fig. 20). Bar = 20 μm for all micrographs.

(A) and (B) one large nucleus (arrow) is present in the cell.

(C) and (D) Two separated nuclei (arrows) are present in the cell.

(E) and (F) Two nuclei (arrows) in contact with each other in one Type 2 stomata.

Also note that a Type 4 stoma is nearby (arrowheads).
Figure 30.
Figure 31. Nuclear number in cyd Type 3 stomata.

DAPI fluorescence. (A), (C), and (E) are the bright-field images corresponding to (B), (D), and (F), respectively. One or two nuclei per cell were found. Nuclear size varies, with single nuclei tending to be larger. Bar = 20 μm for all micrographs.

(A) and (B) A Type 3 stoma with a single cell wall protrusion (arrowhead) that contains one nucleus (arrow).

(C) and (D) A Type 3 stoma with a single cell wall protrusion (arrowhead) that contains two nuclei in contact with each other (arrows).

(E) and (F) A Type 3 stoma with two cell wall protrusions (arrowheads) that contains two separated nuclei (arrows).
Figure 31.
Figure 32. Nuclear number in a *cyd* Type 4 stoma.

(B) is the bright-field image corresponding to DAPI fluorescence in (A). Two separated nuclei (arrows) are present in the cell. No Type 4 stomata had only one nucleus. Bar = 20 µm for both micrographs.
Figure 33. Percentage of Types 2-4 abnormal stomata with one or two nuclei in cyd.

Data are based upon analysis of DAPI fluorescence. Three types (2-4) of abnormal stomata in cyd (see Fig. 19) were analyzed. It appears that the proportion of cells containing two nuclei increases along with greater cell partitioning. All Type 4 stomata had two nuclei. Numbers of cells scored: Type 2 = 70, Type 3 = 30, Type 4 = 25.
Figure 33. Types of Abnormal Stomata

- □ 1 nucleus per cell
- □ 2 nuclei per cell

Percent of Cells
Figure 34. Pollen size in wild type and \textit{cyd} plants.

The product of the polar diameter and the equatorial diameter of the pollen grain was used as an approximate index of pollen size for a population of wild-type \((n = 60)\) and \textit{cyd} \((n = 120)\) pollen grains (see Materials and Methods). The size classes are expressed as a percent of all grains. \textit{cyd} contains much larger pollen than the wild type.
Figure 35. Examples of abnormal vegetative growth of *cyc* seedlings.

Seed were sown singly on agar medium in gridded dishes in different conditions (all four possible combinations of 22°C or 30°C and 30 or 100 μmol m⁻² s⁻¹ light intensity). 8-10 day old *cyc* seedlings (108-122 plants for each set of cultural conditions) were examined and photographed using a dissecting microscope. At this stage, *cyc* seedlings rarely had a wild-type-like morphology. Instead they exhibited various abnormalities, which are assigned to four classes (see text). Examples of 8-10 day old Class 2-4 plants are shown. Bars = 10 mm in (A), (B), (E), 3 mm in (D), and 1 mm in (C) and (F).

(A) A 10 day old wild-type seedling.

(B) This Class 4 seedling is relatively healthy. But note that the first two leaves have unequal sizes (arrows) and more pronounced teeth (arrowheads), and the cotyledons are not as round as in the wild type.

(C) A Class 2 plant with only one cotyledon that does not appear to have a root.

(D) A Class 2 plant with some root development and perhaps one oddly-shaped cotyledon.

(E) A Class 2 plant with a relatively normal root and a single cotyledon, and perhaps some small leaves.

(F) A Class 3 plant with a red, spine-like structure located in the approximate position of a leaf. The petioles of two cotyledons are visible (arrows).
Figure 35.
Figure 36. Vegetative organ fusion, lobing and unusual extra structures in cyd plants.

Methods as in Fig. 35. Shown are 8-10 day old Class 3 plants. Bars = 1 mm in (A), and 5 mm in (B) to (D).

(A) Fused petioles of two cotyledons.

(B) Cotyledon lobing (arrow) and an unusual extra structure growing out of the region of the shoot tip (arrowhead).

(C) Shows either the lobing of one of the first pair of leaves (arrow), or alternatively, the fusion of two leaves belonging to the first and second pairs (from different plastochrons).

(D) A tiny adventitious shoot (arrow) connected to a “parent” seedling by “cord” tissue (arrowhead) projecting from the parent shoot tip. Leaves of the parent seedling were removed to show the phenotype.
Figure 36.
Figure 37. Percentage of all cyp seedlings with different vegetative abnormalities.

The percentages of the four classes of abnormalities of all cyp plants are shown for each of the four different cultural conditions. Seedlings were scored when 8-10 days old. The most Class 4 seedlings were produced at 22°C and 30 μmol m⁻² s⁻¹ light intensity. But overall, all four sets of cultural conditions resulted in the same range and somewhat comparable percentages of abnormalities.
Figure 37.
Figure 38. Percentage of flowers with various abnormalities in cyd plants.

*cyd* flower development was examined in plants exhibiting Class 3 and 4 vegetative abnormalities (Figs. 35-37) 30-40 days after transfer to pot culture (22 ± 1°C or 30 ± 2°C, 50 μmol m⁻² s⁻¹ light intensity). Percentages of different abnormal flowers of all the flowers (total of 246) were calculated. More than fourteen different types of abnormalities were scored and numbered in order of their frequency of occurrence:

1. five stamens,
2. two fused fused sepals,
3. fusion of a stamen and a carpel,
4. fusion of a stamen and a petal,
5. two fused stamens,
6. five normal and one reduced stamens,
7. four stamens,
8. five stamens plus 1 reduced stamen and another stamen-like structure or amorphous tissue,
9. three fused sepals,
10. one reduced petal,
11. three fused stamens,
12. two fused petals,
(13) deformed carpels,

(14) miscellaneous other anormalities.
Figure 38.

Percent of All Abnormalities

Floral Organs with Various Abnormalities

0 5 10 15 20 25 30 %
Figure 39. Fusion between the same type of floral organ or parts of a stamen in *cyd* plants.

Sampling as in Fig. 38. Bars = 2 mm in all micrographs.

(A) Shows two fused sepals, a fusion which usually occurs at the base.

(B) Three fused sepals.

(C) Two fused petals.

(D) Two pairs of fused stamens found in the same flower. The extent of fusion varies.

(E) Three fused stamens. Two are fused along their entire length, and one is fused mostly at its base.

(F) Four fused stamens. The two central stamens have the greatest extent of fusion.

(G) One anther appears to be split and one half of it is fused with the filament (arrow).
Figure 39.
Figure 40. Fusion between different floral organs or organ parts in *cyd* plants.

Sampling as in Fig. 38. Bars = 2 mm in (A), (B) and (D), and 1 mm in (C) and (E).

(A) Fusion of a sepal (arrow) and a petal (arrowhead).

(B) A stamen (arrow) fused with a petal. The anther contains pollen.

(C) A stamen fused with a carpel along the entire length of the filament.

(D) A stamen fused with a carpel at the base (arrow) of the filament.

(E) An anther fused with a partial petal which has papillar cells (arrowheads) characteristic of a normal petal. Also note that despite fusion, the anther still produced pollen (arrow).
Figure 40.
Figure 41. Missing or reduced floral organs in cyd plants.

Sampling as in Fig. 38. Bars = 2 mm in (A) to (E), and 1 mm in (F).

(A) Four stamens plus a reduced fifth one (arrow) from the same flower.

(B) Flower with five stamens.

(C) Flower with five stamens plus a filament-like structure which is forked (arrow).

(D) Flower with a reduced petal (arrow).

(E) Flower with five normal-sized stamens plus two adjacent reduced stamen-like structures (arrows).

(F) A reduced stamen (arrow) is associated with amorphous tissue (arrowhead).
Figure 42. Percentage of normal and abnormal flowers from *cyl* plants whose seedlings exhibited Class 3 and 4 vegetative abnormalities.

Sampling as in Fig. 38. Sample sizes: (1) 22°C, 84 flowers from 19 Class 3 plants and 113 flowers from 22 Class 4 plants; (2) 30°C, 55 flowers from 17 Class 3 plants and 102 flowers from 26 Class 4 plants. The results are similar for all four groups of plants regardless of their earlier growth forms and later temperatures.
Figure 42.
Figure 43. Percentage of normal and abnormal flowers in *cyd* plants from seedlings first grown at 30 or 100 μmol m⁻² s⁻¹ light intensity.

Data from Fig. 42 regrouped according to light intensity (30 or 100 μmol m⁻² s⁻¹) experienced by seedlings during their first 10 days of growth. Sample size: (1) 22°C: 98 flowers from 21 plants initially grown at 30 μmol m⁻² s⁻¹ light intensity, and 99 flowers from 20 plants initially grown at 100 μmol m⁻² s⁻¹ light intensity; (2) 30°C: 75 flowers from 21 plants initially grown at 30 μmol m⁻² s⁻¹ light intensity, and 82 flowers from 22 plants initially grown at 100 μmol m⁻² s⁻¹ light intensity. The higher light intensity during the first 10 days of growth of the seedlings promoted the production of normal flowers.
Figure 43.
LIST OF REFERENCES


APPENDIX A

PROTOCOLS FOR CAPS MAPPING

DNA Minipreparation

1. 10-15 F$_3$ seedlings (15 days old) or individual adult F$_2$ plants are frozen in liquid nitrogen, and ground to powder in a 1.7 ml microcentrifuge tube with a pestle.

2. Extraction buffer (500 µl) containing 100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl and 10 mM (0.35 µl) mercaptoethanol is added to the tube followed by the addition of 35 µl of 20% SDS.

3. The sample is incubated at 65°C for 10 minutes and 130 µl of 5 M potassium acetate (KAc) is added, then the sample is incubated at 0°C for 5 minutes.

4. The precipitation is pelleted for 10 minutes at 15,000 g.

5. The supernatant is transferred to a 1.7 ml tube containing 640 µl isopropyl alcohol, and 60 µl 3 M sodium acetate (NaAc, pH 5.2), mixed, and incubated at -20°C for 10 minutes.

6. The precipitated DNA is centrifuged at 15,000 g for 15 minutes and redissolved in 200 µl of 50 mM Tris pH 8, 10 mM EDTA.

7. This solution is centrifuged at 15,000 g for 5 minutes to remove insoluble
materials, and the supernatant is transferred to a tube containing 20 µl 3 M NaAc and 440 µl ethanol.

8. This tube is incubated at -20°C for 10 minutes. The DNA is pelleted by centrifugation at 15,000 g for 5 minutes, and washed with 200 µl 70% ethanol. Pellets are centrifuged again for 5 minutes, dried and dissolved in 50 µl water.

**Polymerase Chain Reaction (PCR) Amplification**

PCR is carried out in a 10 µl final volume which contains 0.125 mM each of four deoxynucleotides, 40 ng of both primers, 0.25 U of Taq polymerase, and 1 µl of the 50 µl DNA obtained from a miniprep (about 50-100 ng of DNA). Conditions for the amplification are 30 seconds at 95°C, annealing for 30 seconds at 56°C, and polymerization for 3 minutes at 72°C. The cycle is repeated 50 times.

Usually a premix containing deoxynucleotides, Taq polymerase buffer, primers and Taq polymerase is prepared for 30-40 reactions and transferred to 0.5 ml microcentrifuge tubes to which various DNA samples are added.

**Enzyme Digestion and Analysis of PCR Products**

Restriction endonuclease digestion is carried out in the same tube as the PCR reaction. restriction enzyme mix (10 µl) containing 2 µl of restriction enzyme buffer and 15 U of Enzyme is added to each tube and incubated for 2 hours at the temperature optimal for the activity of that particular enzyme. 3 µl of 6x loading dye is added to each tube. The digestion products are analyzed on 1.5% agarose gels. DNA of Landsberg and
Columbia parents separately or in an equal mixture are run in adjacent lanes to visualize polymorphisms.