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The *CURLY LEAF* gene controls both division and elongation of cells during the expansion of the leaf blade in *Arabidopsis thaliana*

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Abstract. The *CURLY LEAF* (*CLF*) gene in *Arabidopsis thaliana* (L.) Heynh. is required for stable repression of a floral homeotic gene, *AGAMOUS* in leaves and stems To clarify the function of *CLF* in organ development, we characterized *clf* mutants using an anatomical and genetic approach. The *clf* mutants had normal roots, hypocotyls, and cotyledons, but the foliage leaves and the stems had reduced dimensions. A decrease both in the extent of cell elongation and in the number of cells was evident in the *clf* mutant leaves, suggesting that the *CLF* gene might be involved in the division and elongation of cells during leaf morphogenesis. An analysis of the development of *clf* mutant leaves revealed that the period during which cell division or cell elongation occurred was of normal duration, while the rates of both cell production and cell elongation were lower than in the wild type. Two phases in the elongation of cells were also recognized from this analysis. From analysis of an *angustifolia clf* double mutant, we found that the two phases of elongation of leaf cells were regulated independently by each gene. Thus, the *CLF* gene appears to affect cell division at an earlier stage and cell elongation throughout the development of leaf primordia.

Key words: *Arabidopsis* (leaf morphogenesis) – Cell division – Cell elongation – *CURLY LEAF* – Leaf blade expansion

Introduction

The morphology of multicellular organisms is attributable to mechanisms that regulate the shapes, sizes, and numbers of the constituent cells. In higher animals, the body plan is basically established at the stage of gastrulation. By contrast, in plants, the body plan is not strictly determined and, throughout the life cycle of the plant, new organs are added to the body via meristems located at the apices of the roots and shoots. The fundamental unit of each vegetative shoot system can be considered to consist of a leaf, an internode, and a lateral bud.

Our current goal is the identification of the various genes that control the development of the leaf, a fundamental component of the shoot. The rates of division and elongation of cells, at each stage, are known to contribute to the final shape of the leaf (Arkebuer and Norman 1995; Maksymowych 1963; Sunderland 1960) and to play important roles throughout leaf development. Nonetheless, the mechanisms controlling these basic aspects of leaf development remain to be characterized since the pattern of growth within the leaf blade is quite complex (Poethig and Sussex 1985; Steeves and Sussex 1989).

In our efforts to dissect the complicated process of leaf morphogenesis, we have focused on genetic analysis of the model plant *Arabidopsis thaliana* (L.) Heynh. (Tsukaya 1995). We have found that the two-dimensional growth of the leaf blade is genetically controlled through regulation of the polar elongation of cells (Tsukaya et al. 1994; Tsuge et al. 1996). The present report describes the results of our investigations of the development of leaf cells of wild-type *A. thaliana* and of the *curly leaf* (*clf*) mutant, which has leaves with unusual morphology (Coupland et al. 1993; Sieburth and Meyerowitz 1997).

The *CLF* gene is a member of the polycomb-group of genes which is known for regulation of homeotic gene expression (Jones and Gelbart 1993; Kennison 1993) and is necessary for the stable repression of a floral homeotic gene, *AGAMOUS* (*AG*; Goodrich et al. 1997). The *clf* phenotype, namely, narrow, small and curled leaves, is very similar to that of transgenic plants in which the *AG* gene is expressed constitutively (Mizukami and Ma 1992), and it seems possible that

Abbreviations: *clf* = *curly leaf*; *AG* = *AGAMOUS*; *an* = *angustifolia*; Col = Columbia; En2 = Enkheim2

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the *clf* phenotype might be the result of misexpression of *AG*. The pattern of expression of the *CLF* gene in wild-type plants (Goodrich et al. 1997) suggests that *CLF* mRNA accumulates in leaf primordia, apical meristems, and the vasculature of cotyledons and hypocotyls. Recent studies also suggest that the wild-type *CLF* gene is required to repress transcription of the *AG* gene in leaves, inflorescence stems and flowers (Goodrich et al. 1997; Sieburth and Meyerowitz 1997). The *AG* gene is speculated to be responsible for control of cell proliferation in floral morphogenesis (Meyerowitz 1997). However, the function of the *CLF* gene in cell proliferation remains to be clarified.

Our analysis of the leaves of *clf* mutant plants has allowed us to identify a gene that appears to regulate both the division and the elongation of cells during leaf development. Our analysis also indicates that the elongation of leaf cells can be divided into two distinct phases.

Materials and methods

Sources of mutant alleles and strains. The following plants were used: *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col); Enkheim2 (En2); seven *curly leaf* (*clf*) mutant strains, and one *angustifolia* (*an*) mutant strain. The seven *clf* mutants, namely, F43, F67, F68, F69, F70, F72, and F150, were originally identified among mutants collected by Dr. A.R. Kranz (En2 ecotype; supplied by Sendai Seed Stock Center, Sendai, Japan). These mutant strains all had mutations at the same locus, as demonstrated by an allelism test in this study that involved crosses between the various strains. These mutants, which were isolated on an En2 background, were back-crossed twice to Col wild type to allow analysis of the phenotype in a comparison with strains with the Col background. The En2 wild type was also analyzed in order to estimate the effects of the genetic background. All the back-crossed mutants had narrow, rolled up, short leaves, and early flowering, as compared with wild-type Col. An allelism test was also performed on a line with a similar phenotype, due to the mutant *clf-81* allele, which had been isolated and kindly donated by Dr. W.-D. Reiter (University of Connecticut). It was derived from ethyl methanesulfonate-mutagenized lines of Col ecotype. The F150 mutant was crossed with the *clf-81* mutant and the F_1 and F_2 progeny were analyzed. All progeny had the same phenotype as that of the F150 mutant. Thus, the F150 mutation appeared to be allelic to the *clf* mutation. The research group of Dr. E.M. Meyerowitz also identified these strains as being allelic to the *clf* mutant. Thus, the back-crossed strains were further identified as F43, *clf-17*; F67, *clf-18*; F68, *clf-21*; F70, *clf-5*; F72, *clf-23*; F75, *clf-24*; and F150, *clf-25* (Dr. E.M. Meyerowitz, personal communication). The *clf-25* mutant, which had the most extreme phenotype among the seven *clf* mutant strains identified in this study, was chosen for further detailed study. To avoid any effects of the genetic background, the *clf-25* mutant strain was back-crossed twice to the Col wild type before detailed analysis and was also compared with the En2 wild type.

Growth of plants. Plants were grown in small plastic pots with rockwool and/or vermiculite that had been moistened with MGRL medium (Tsukaya et al. 1991) at 22 °C under continuous illumination from fluorescent lamps, as described previously (Tsuge et al. 1996).

Dimensions of fully extended shoots at each position (type 2 metamer; Schultz and Haughn 1991) were measured after growth under the conditions described above. For analysis of the growth of roots and hypocotyls, plants were cultured on agar-solidified medium (Okada and Shimura 1992). For cultivation of plants on plates, seeds were surface-sterilized as described previously (Tsukaya et al. 1991), and then they were washed extensively with sterilized water. The plates were incubated vertically for observation of the growth of roots and hypocotyls, under the conditions described above.

Measurements and anatomy of leaves. Leaves at each position in the shoot system were analyzed after growth in pots under the conditions described above. The leaves were numbered from the first rosette leaf that emerged after the cotyledons to the last rosette leaf. In this study, the appearance of a leaf 1 mm long was defined as the initiation of a leaf primordium. The definitions of directions within each leaf blade were those originally provided by Tsuge et al. (1996).

For anatomical analysis, samples were embedded in Technovit 7100 resin (Kulzer & Co., Wehrheim, FRG) and examined as described previously (Tsukaya et al. 1993). For transverse sections, samples were cut at the center of the fifth rosette leaf blade. Longitudinal sections were prepared by cutting samples along the midrib of the leaf blade (Tsuge et al. 1996).

Observations of epidermal cells. Samples of leaves and cotyledons were fixed in FAA solution [5% (v/v) acetic acid, 45% (v/v) ethanol, and 5% (v/v) formaldehyde] under a vacuum and rendered transparent by incubation overnight in a solution of chloral hydrate (chloral hydrate, 200 g; glycerol, 20 g; H_2O, 50 mL) as described by Tsuge et al. (1996). Then samples were stained with a 0.1% (w/v) solution of toluidine blue in 0.1 M sodium phosphate buffer (pH 7.0) for 2 min. Samples were photographed under bright-field illumination to obtain paradermal images of the layers of leaf cells. The average epidermal cell area was determined by measuring the total area of epidermal cells on a photograph and then dividing this area by the number of epidermal cells in the photograph.

Results

To dissect the complex developmental processes involved in leaf expansion, we have isolated and examined mutants of *A. thaliana* with defects in these processes. In this study, we focused on *clf* mutants, which have narrow, curled leaf blades of reduced length (Fig. 1A).

The CLF gene affects the morphology and the expansion of leaves from a specific stage of leaf development. When grown under constant illumination, each *clf-25* mutant plant produced an average of two cotyledons, nine rosette leaves and three cauline leaves, which are about the same numbers as made by the wild type (Fig. 1B). All the foliage leaves of the *clf-25* and *clf-81* mutants were narrow, short and curled upwards, as shown in Fig. 1. The morphology of the cotyledons of the *clf-25* mutant was similar (Fig. 1B; Table 1) to that of the wild type. The leaves of wild-type Col and those of wild-type En2, from which *clf-25* was originally derived, were similar (data not shown), which suggests that the altered morphology of *clf-25* mutant did not result from the difference in genetic background between the two ecotypes.

Among the rosette leaves, the fifth has the most reproducible distinguishing features of the Col wild type (Fig. 1B; Tsuge et al. 1996). Therefore, for further analysis, we chose the fifth rosette leaf. To determine the developmental stage at which the *CLF* gene affected

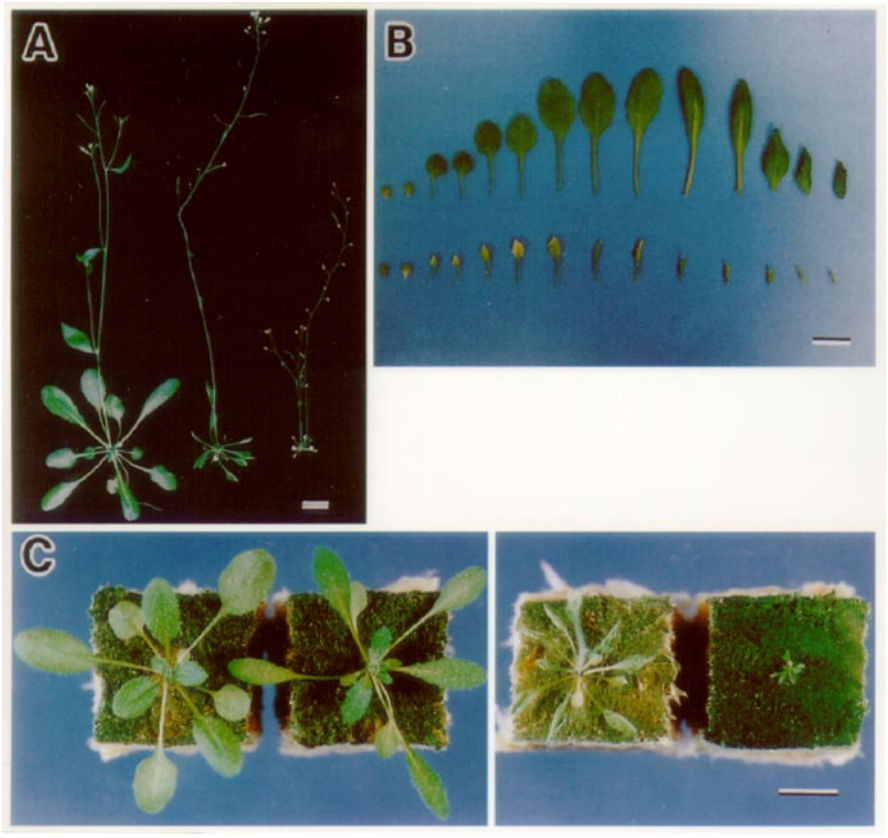

Fig. 1A–C. Morphology of *Arabidopsis thaliana* plants. **A** The gross morphology of wild-type, *clf-25* and *clf-81* plants, from left to right. The plants were photographed 4 weeks after sowing of seeds. **B** Leaves of the wild type (*upper row*) and the *clf-25* mutant (*lower row*). The leaves in each row are, from the left, the two cotyledons, the nine rosette leaves, and the three cauline leaves. The leaves were collected when fully expanded. **C** The morphology of wild-type, *an*, *clf-25* and *an clf-25* double mutant plants, from left to right. The plants were photographed 3 weeks after sowing of seeds. Bars = 1 cm

morphogenesis of the leaf blade, we analyzed the growth of wild-type and *clf-25* mutant plants in terms of the width and the length of the fifth leaf. The fifth leaf primordium (a leaf 1 mm long was observed, according to the above definition) was initiated earlier in the *clf-25* mutant than in the wild type. By contrast, the expansion of the fifth leaf of the *clf-25* mutant was complete within 15 to 18 d after sowing, whereas that of the wild-type leaf continued beyond this time. In addition, we also examined the flowering time of the wild type and the *clf-25* mutant. The flowering time of the *clf-25* mutant was ca. 3 d earlier than that of the wild type under continuous illumination.

The clf mutation caused a decrease in both the number and the size of leaf cells. To identify the roles of cell expansion and cell production in the phenotype of the leaf blade of the *clf-25* mutant, we compared the mature anatomy of the fifth rosette leaf with that of the wild type. The first layer of palisade cells (subepidermal cells) consisted of an organized arrangement of neatly aligned cells, and this layer was used for the enumeration and

Table 1. Dimensions of wild-type (wt) and *clf-25* mutant *Arabidopsis* plants. Data are means ± SD for *n* plants examined

Organ[a]	mm	
	wt (Col)	*clf-25*
Cotyledon width	2.36 ± 0.28 (n = 10)	2.26 ± 0.33 (n = 10)
Cotyledon length	2.64 ± 0.19 (n = 10)	2.59 ± 0.28 (n = 10)
Hypocotyl length[b]	1.90 ± 0.37 (n = 25)	1.97 ± 0.33 (n = 29)
Leaf width[c]	9.1 ± 0.7 (n = 6)	3.1 ± 0.2 (n = 5)
Leaf length[c]	14.3 ± 0.8 (n = 6)	5.5 ± 0.4 (n = 5)
Internode diameter	12.1 ± 0.4 (n = 6)	7.1 ± 0.2 (n = 4)
Internode length		
Type 2 metamer (1st)	41.3 ± 9.5 (n = 4)	35.6 ± 9.3 (n = 5)
Type 2 metamer (2nd)	123.8 ± 19.3 (n = 4)	81.2 ± 7.6 (n = 5)
Total length[d]	262.5 ± 25.9 (n = 4)	242.8 ± 8.3 (n = 5)

[a]Cotyledons and fifth rosette leaves were measured at the fully expanded stage, and hypocotyls and internodes were measured at the mature stage
[b]Plants were grown on agar plates and then hypocotyls were measured
[c]Measurements were made on sections
[d]Type 2 metamer + type 3 metamer

Table 2. Dimensions of palisade cells (first layer) in wild-type (wt) and *clf-25* mutant *Arabidopsis* plants. Data are means ± SD for more than 150 cells from *n* plants

Direction[a]	μm	
	wt (Col)	*clf-25*
Leaf-width direction	40.6 ± 3.3 (n = 5)	24.1 ± 1.3 (n = 4)
Leaf-length direction	42.6 ± 2.2 (n = 4)	25.2 ± 2.1 (n = 3)
Leaf-thickness direction	32.0 ± 7.6 (n = 5)	20.8 ± 6.8 (n = 3)

[a] Measurements were made on sections

characterization of cells in both the leaf-width and the leaf-length directions. In transverse sections, the width of palisade cells of the *clf-25* mutant was 59% of that of the wild type in the fully expanded fifth leaf (Table 2). In longitudinal sections, the length and thickness of palisade cells of the *clf-25* mutant were 59% and 65%, respectively, of those of the wild type at the same stage (Table 2). The number of palisade cells in the leaf blade of the *clf-25* mutant was 55% in the leaf-width direction and 67% in the leaf-length direction, respectively, of the number in the wild type (Table 3). In the sections, the layers of parenchyma cells which aligned in the leaf-thickness direction in the leaf blade of both the *clf-25* mutant and the wild type were four. Thus, the total number of cells in the *clf-25* leaf blade was also significantly smaller than that in the wild-type leaf blade (Table 3). We performed two back-crosses of the *clf-25* mutant with the Col wild type before analysis. However, in order to estimate the effects of the En2 genetic background, we also performed an anatomical analysis of En2 and compared wild-type En2 with wild-type Col. The cells of the fifth leaf in wild-type En2 did not differ in number and size from those in wild-type Col (data not shown). These anatomical results suggest that a reduction in both the size and the number of cells was responsible for the altered leaf morphology in the *clf-25* mutant.

It is generally considered that epidermal cells have an important role in the process of leaf expansion (Dale 1988). Therefore, we postulated that the epidermal cells might have unique features in the *clf* mutant. Most of the epidermal cells on the abaxial and adaxial sides had a complex jigsaw-like shape with many protrusions in various directions (Fig. 2). The shapes of epidermal cells of the *clf-25* mutant were slightly different from those of the wild type (Fig. 2). The surface area of each epidermal cell in the *clf-25* mutant was reduced on both the abaxial and the adaxial sides, as compared to the wild type (Table 4). Thus, a reduction in the surface area of epidermal cells was involved in the altered leaf morphology of the *clf-25* mutant, in addition to the changes in the palisade cells.

The rates of cell production and cell elongation in the clf mutant differed from those in the wild type during leaf development. To determine the function of the *CLF* gene in the regulation of the development of leaf cells, we analyzed the temporal patterns of production and elongation of cells in the *clf* mutant and the wild type (Fig. 3). Since the initiation of the primordium of the fifth leaf (defined as the appearance of a leaf 1 mm long) in the *clf-25* mutant occurred earlier than in the wild type, temporal patterns of leaf development were analyzed after the appearance of the leaf primordium. The growth of the leaf of the *clf-25* mutant was slower than that of the wild type throughout its development (Fig. 3A, B). Four days after the appearance of the primordium, the division of palisade cells ceased in both the wild type and the *clf-25* mutant. The rate of cell production was lower in the *clf-25* mutant than in the wild type throughout the period of cell division after the appearance of the leaf primordium (Fig. 3C,D). During the elongation of leaf cells, two phases could be recognized. While cells were dividing, the size of cells in the *clf-25* mutant did not differ from that in the wild type (Fig. 3E,F). After the completion of the cell-division phase (8 d after the appearance of the leaf primordium), in the observed area of the leaf blade, expansion continued in both the wild type and the *clf-25* mutant. The rate of cell elongation at this stage was lower in the *clf-25* mutant than in the wild type (Fig. 3E,F). These data show that the rates of both the production and the elongation of cells were lower in the

Table 3. Anatomical analysis of the fifth rosette leaves of wild-type (wt) and *clf-25* mutant *Arabidopsis* plants. Data are means ± SD from *n* plants examined

	Number of leaf cells	
	wt (Col)	*clf-25*
Cells aligned in a transverse section		
Palisade cells[a]	216 ± 30.8 (n = 5)	119 ± 4.2 (n = 4)
Total cells[b]	721 ± 96.8 (n = 3)	280 ± 24.2 (n = 4)
Cells aligned in a longitudinal section		
Palisade cells[a]	261 ± 21.3 (n = 4)	174 ± 9.5 (n = 3)
Total cells[b]	808 ± 168.4 (n = 3)	482 ± 82.2 (n = 3)

[a] Cell numbers in the first layer of palisade cells aligned in each direction were analyzed
[b] Total numbers of cells in sections in each direction, excluding the cells in the epidermal layer, the xylem and the phloem, were analyzed

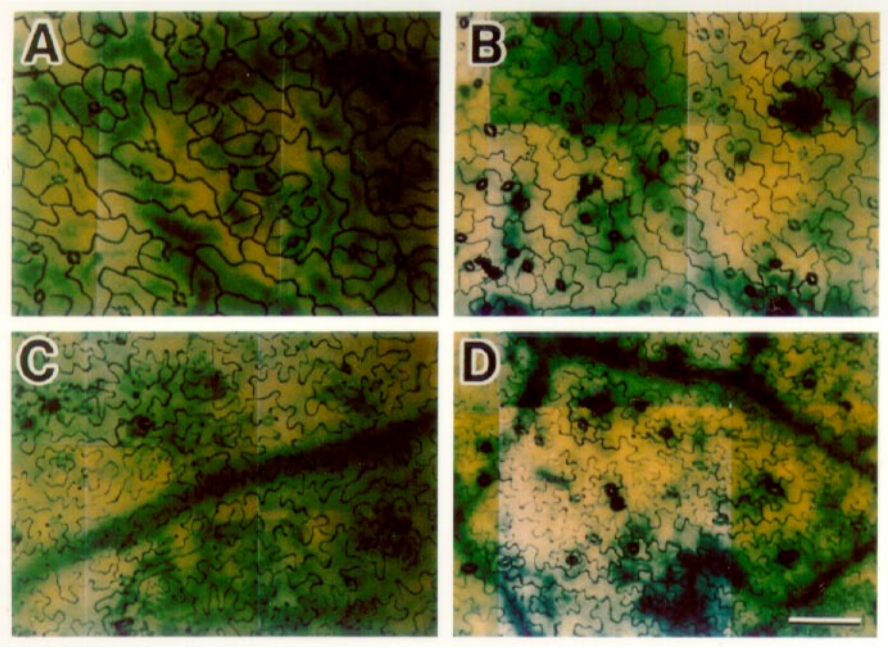

Fig. 2A–D. Epidermal cells in the fifth leaf blade of wild-type and *clf-25 Arabidopsis* plants. **A, B** Adaxial surface of a wild-type leaf (**A**) and of a *clf-25* leaf (**B**). **C, D** Abaxial surface of a wild-type leaf (**C**) and of a *clf-25* leaf (**D**). The direction perpendicular to the images in the figure corresponds to the long axes of the leaves. The leaves were photographed at the fully expanded stage. Bar = 100 μm

Table 4. Analysis of the area of epidermal cells in the fifth leaves of wild-type (wt) and *clf-25* mutant *Arabidopsis* plants. Data are means ± SD for more than 92 cells per leaf. Numbers in parentheses show the ratio of area of epidermal cells in *clf-25* to that in the wild type

	wt (Col)	*clf-25*
Adaxial cell[a] (μm^2)	3923 ± 523.9 (1)	1594 ± 373.9 (0.41)
Abaxial cell[a] (μm^2)	3012 ± 384.7 (1)	1302 ± 111.3 (0.43)

[a]Guard cells were excluded from measurements. Four leaves were examined for each measurement

clf mutant than in the wild type, but the durations of the cell-division phase and the cell-elongation phase during leaf development were unchanged in the *clf* mutant.

The CLF gene does not influence the polarity-specific elongation of leaf cells. In order to determine the role of the *CLF* gene within the genetic framework of leaf development, we introduced the *an* mutation, which affects the polarity-specific elongation of leaf cells (Tsuge et al. 1996), into the *clf* mutant. Figure 1C shows the phenotypes of the wild type, the *an* mutant, the *clf-25* mutant and the *an clf-25* double mutant. The growth of the fifth leaf of the *an clf-25* double mutant was reduced in both the leaf-length and the leaf-width directions, as compared to the wild type and both single mutants (Fig. 1C). The *an clf-25* double mutant had fewer cells in the leaf-width and leaf-length directions than either single mutant. The cells themselves were also smaller in the leaf-width and leaf-length directions than in either single mutant. In the leaf-thickness direction, the length of cells was intermediate between those of the two single mutants (Table 5). Therefore, the *an* mutation and the *clf* mutation had an additive effect.

These results suggest that the regulation by the *CLF* gene of the division and elongation of cells is independent of the polarity-specific regulation of the elongation of cells in leaves by the *AN* gene.

The number of internode cells was reduced in the clf-25 mutant. In order to determine the organ specificity of the action of the *CLF* gene, we also examined the dimensions of organs other than leaves. The average length of hypocotyls of *clf-25* plants, after growth from seeds for 9 d on agar plates, did not differ from that of wild-type plants (Table 1). The growth rate of the primary roots of *clf-25* mutant plants was also not significantly different from that of the wild type (Fig. 4). Thus, no defects were found in the length of the hypocotyl or of the primary root.

The internodes of floral stems of the *clf-25* mutant were shorter than those of the wild type at maturity (Table 1). The internodes were also thinner than those of the wild type (Fig. 5; Table 1). To clarify whether this defect was accompanied by a decrease in cell number or a decrease in cell size, we performed an anatomical study of sections of first internodes of type 2 metamers (that part of the primary shoot that bears secondary inflorescences and cauline leaves; Schultz and Haughn 1991) from the wild type and the *clf-25* mutant. The diameter and length of pith cells in the *clf-25* mutant were not very different from those in the wild type (Fig. 5). However, the shoots of the *clf-25* mutant contained only 46% of the wild-type number of cells (Table 6). These results show that cell numbers were also changed by the *clf* mutation in the internodes of floral stems.

Discussion

The CLF gene appears to play an important role in shoot development. As summarized in the *Introduction*, molecular characterization of the *CLF* gene has been carried out to understand control of *AG* gene expression

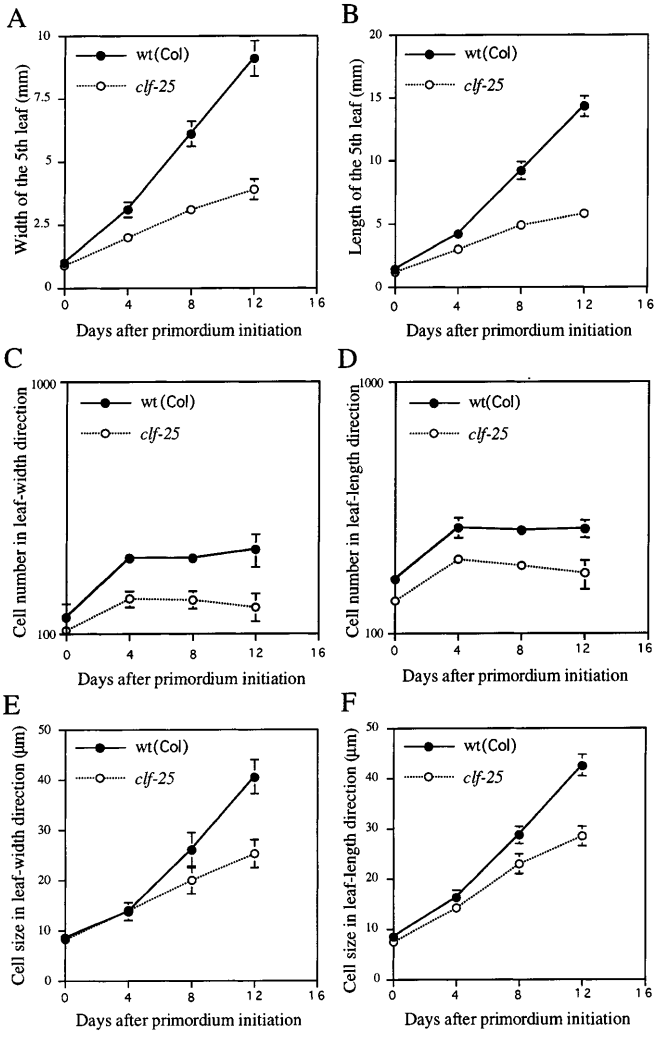

Fig. 3A–F. Growth of the fifth rosette leaves of wild-type(wt) *Arabidopsis* and the *clf-25* mutant. **A, B** Increase in leaf width (**A**) and length (**B**). **C, D** Logarithmic plot of cell number in the leaf-width direction (**C**) and in the leaf-length direction (**D**) for palisade cells vs. days after initiation of leaf primordia. **E, F** Changes in cell dimensions in the leaf-width direction (**E**) and in the leaf-length direction (**F**) for palisade cells

(Goodrich et al. 1997). The *AG* gene is speculated to be needed for proper control of cell proliferation in flower development (Meyerowitz 1997). However, detailed

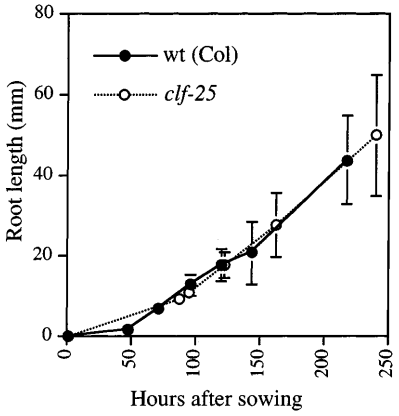

Fig. 4. Growth of roots of the wild-type (*wt*) *Arabidopsis* and the *clf-25* mutant. See text for details

morphological analysis of the *clf* mutants had not been performed to date.

The leaves of *clf* were consistently the most severely affected organ. The leaves of *clf-25* plants were narrow and curled upwards along the longitudinal axis of the leaf (Fig. 1). What makes the leaves of *clf* mutants curl upwards along the leaf margin? Two possibilities can be considered . One is that the reduction in cell expansion is greater along the leaf margin than along the leaf midrib. The other is that the reduction in cell division is greater in the upper layer of the leaf cells than in the lower layers. Although we could not determine which of these possibilities is correct, due to the complexity of the irregular-shaped spongy cells and epidermal cells, the expansion of cells in the leaf margin did not differ from that along the leaf midrib (data not shown).

From our analysis, a decrease in numbers of cells was observed in both the foliage leaves and the stems. In view of the organ specificity of the mutant phenotype, it seems that, in *A. thaliana*, the *CLF* gene is necessary during various stages of shoot development but not in the development of roots or embryonic organs, such as the cotyledons and hypocotyls. This organ-specific phenotype corresponds to the results of localization studies of *CLF* mRNA, which suggested that *CLF* is expressed not only in leaf primordia, meristems and the vasculature of cotyledons and hypocotyls, but also in the leaves and flowers at various stages of plant development

Table 5. Anatomical analysis of the fifth rosette leaves of *an*, *clf-25* and *an clf-25* double mutant *Arabidopsis* plants. Data are means ± SD for more than 150 cells from *n* plants

	an	*clf-25*	*an clf-25*
Number of palisade cells aligned in[a]			
the leaf-width direction	156 ± 12.2 (n = 3)	119 ± 4.2 (n = 4)	66 ± 4.4 (n = 4)
the leaf-length direction	272 ± 9.1 (n = 3)	174 ± 9.5 (n = 3)	142 ± 23.1 (n = 3)
Dimensions (μm) of palisade cells in[b]			
the leaf-width direction	33.8 ± 1.6 (n = 3)	24.1 ± 1.3 (n = 4)	17.8 ± 3.3 (n = 5)
the leaf-length direction	41.8 ± 2.7 (n = 3)	25.2 ± 2.1 (n = 3)	18.6 ± 4.2 (n = 4)
the leaf-thickness direction	54.5 ± 9.2 (n = 3)	20.8 ± 6.8 (n = 3)	37.0 ± 4.9 (n = 4)

[a]Numbers of cells in the first layer of palisade cells aligned in each direction were analyzed

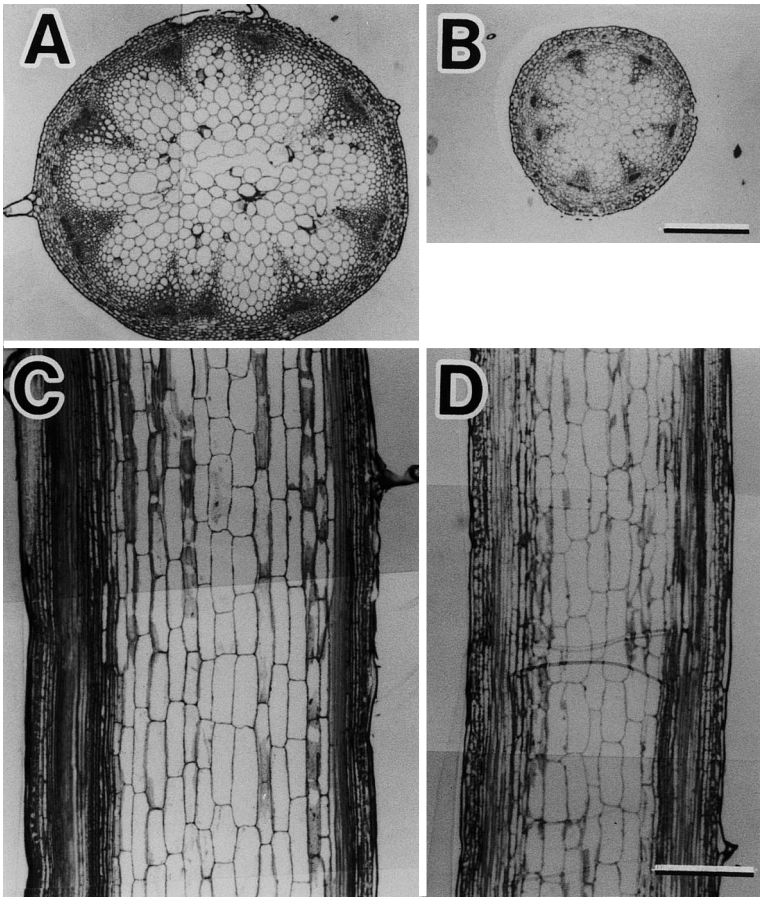

Fig. 5A–D. Internodal sections of wild-type *Arabidopsis* and the *clf-25* mutant. **A, B** Transverse section of a type-2 metamer in the wild type (**A**) and in the *clf-25* mutant (**B**). **C, D** Longitudinal section of a type-2 metamer in the wild type (**C**) and in the *clf-25* mutant (**D**). The samples were prepared from mature plants. Bars = 300 μm

Table 6. Anatomical analysis of shoots of wild-type (wt) and *clf-25* mutant plants. Data are means ± SD for more than 30 cells from each of *n* plants examined

	wt (Col)	*clf-25*
Cells aligned in transverse sections[a]		
Number of cells	296 ± 49.3 (n = 6)	135 ± 12.5 (n = 4)
Area per cell (μm^2)	1543 ± 170	1158 ± 165
Cells aligned in longitudinal sections[a]		
Cell width (μm)	44.3 ± 6.7 (n = 4)	44.6 ± 7.2 (n = 4)
Cell length (μm)	178.6 ± 9.6 (n = 4)	227.7 ± 44.2 (n = 4)

[a]First internodes of type 2 metamers were analyzed. Cortical cells were excluded

(Goodrich et al. 1997). The absence of any defect in the cotyledons of *clf* mutant plants agrees well with the lower-level expression of the *CLF* gene in the cotyledons. It might also reflect a difference in the expansion process between cotyledons and foliage leaves. As will be discussed below, the *CLF* gene appears to affect two distinct phases of the development of foliage leaves. Its function might not be required for the expansion of cotyledons, a process in which cell division does not play an important role in *A. thaliana* (Tsukaya et al. 1994), since the expansion of cotyledons is mostly effected by cell elongation. What, then, is the function of the *CLF* gene in leaf development? It seems likely that these functions of *CLF* in leaf development are an indirect effect of the ectopic expression of floral homeotic genes, such as *AG* and *APETALLA3* (*AP3*). Recently, Goodrich et al. (1997) reported that *ag clf* double mutants had normal leaves, and that expression of floral homeotic genes such as *AG* or *AP3* combined with *PISTILLATA* (*PI*) in leaves of transgenic plants had very similar effects on leaf morphology and plant stature as the *clf* mutation. Although the mechanism of action and the target of the product of the *CLF* gene in leaf development remain unknown, the primary function of *CLF* may be to keep the MADS box genes, such as *AG*, switched off in leaves. Our present study strongly suggests that the *CLF* gene plays an important role in shoot morphogenesis.

The CLF gene might play a role during two phases of leaf development. In an effort to understand the role of the *CLF* gene in leaf development, we examined the timing of various aspects of leaf development in the *clf* mutant and the wild type. Since the timing of the appearance of leaf primordia differed among strains, the appearance of a leaf primordium was defined as the time when a leaf

primordium had reached 1 mm long. The development of the leaf blade can be divided into two phases in terms of the division and elongation of leaf cells: phase A, when both the division and the elongation of cells occur (0–4 d after initiation of a primordium; Fig. 3); and phase B, when cell division has ceased and only the elongation of cells continues. The size of the *clf* mutant cells was significantly smaller in phase B than that of wild-type cells (Fig. 3E, F), which means that the *clf* mutant has a defect in the elongation of leaf cells in phase B. On the other hand, the size of *clf* mutant cells in phase A did not differ from that of wild-type cells (Fig. 3E, F). However, this does not mean that the *clf* mutant has wild-type cell elongation during phase A, since cell size is a balance between cell elongation and cell division. The *clf* mutant showed the normal size of cells (Fig. 3E, F) and the lower rate of cell production (Fig. 3C, D) in the *clf* leaf primordia of phase A. This fact means that the rate of elongation of *clf* mutant cells was also depressed during phase A. We previously divided the process of leaf development into stages I through IV, by reference to the development of leaf primordia (Tsuge et al. 1996). The polar elongation of cells starts during stage II. Thus, the elongation of leaf cells can be divided temporally into stage I and stages II to IV. Phase A, as defined in the present study, corresponds to stages I and II, in terms of the sizes of leaf primordia. Taken together, the results suggest that the elongation of leaf cells can be divided temporally into stage I, stage II, and stages III plus IV. Several independent mechanisms might control the elongation of leaf cells but the *CLF* gene seems to play a role throughout the phases of cell elongation. Analysis of double mutants using the *an* mutation, which results in an altered pattern of polar elongation of leaf cells during phase B (stages III plus IV) as described previously (Tsuge et al. 1996), suggested that the two later-phase aspects of regulation of the elongation of leaf cells were independently controlled (Fig. 1C; Table 5). Recently, Baskin et al. (1995) reported a similar phenomenon in roots of *stunted plant1* (*stp1*) mutants of *A. thaliana*. Baskin et al. (1995) proposed that there are two phases in the elongation of root cells, corresponding to the meristem zone of rapid elongation, and postulated that the product of the *STP1* gene might be essential exclusively during the specific phase when rapid elongation of cells occurs.

In addition to its role in cell elongation during phases A and B, the *CLF* gene also seems to play a role in the division of leaf cells, which occurs during phase A (Fig. 3C). Since the duration of neither the cell-division phase nor the cell-elongation phase was altered by the *clf* mutation, the *CLF* gene appears to affect some activity that is related to both cell division and cell elongation, without any influence on the timing of these two developmental processes.

Several genes have been identified within the genetic framework that controls the expansion of cells in the shoots of *Arabidopsis*. The *DIMINUTO* (= *DWARF1*) gene is thought to be responsible for the elongation of cells in all plant organs, including leaf cells, and encodes an enzyme for the biosynthesis of brassinosteroids (Takahashi et al. 1995; Kauschmann et al. 1996). In shoot development, the *axr1* and *axr2* mutations were reported to affect cell division and cell elongation, respectively (Lincoln et al. 1990; Timpte et al. 1992). The *ACAULIS1* gene appears to be responsible for the elongation of leaf cells and inflorescence cells (Tsukaya et al. 1993), and the *AN* and *ROTUNDIFOLIA3* genes seem to be responsible for the polarity-specific elongation of leaves (Tsuge et al. 1996). However, little is known about regulating both the division and the elongation of cells in leaf development. The *CLF* gene appears to be different from previously reported genes, regulating both the division and the elongation of cells during leaf development. Detailed, molecular analysis of target genes of the *CLF* gene will help us to understand the direct function of the *CLF* gene in leaf morphogenesis.

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