CLAVATA1, a regulator of meristem and flower development in *Arabidopsis*

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SUMMARY

We have investigated the effects on plant development of mutations in the *Arabidopsis thaliana CLAVATA1* gene. In *clavata1* plants, vegetative, inflorescence and floral meristems are all enlarged relative to wild type. The apical meristem can fasciate in the more severe mutant alleles, and this fasciation can occur prior to the transition to flowering. Flowers of *clavata1* plants can have increased numbers of organs in all four whorls, and can also have additional whorls not present in wild-type flowers. Double mutant combinations of *clavata1* with *agamous*, *apetala2*, *apetala3* and *pistillata* indicate that *CLAVATA1* controls the underlying floral meristem structure upon which these homeotic genes act. Double mutant combinations of *clavata1* with *apetala1* and *leafy* indicate *CLAVATA1* plays a role in establishing and maintaining floral meristem identity, in addition to its role in controlling meristem size. In support of this, RNA expression patterns of *AGAMOUS* and *APETALA1* are altered in *clavata1* flowers.

Key words: fasciation, flower, meristem, inflorescence, *Arabidopsis, CLAVATA1*

INTRODUCTION

Two key developmental processes help determine the overall aerial structure of plants. The first process is the establishment of the apical meristem structure, including the positioning of developing primordia. The second process is the specification of the identity of these developing primordia. In *Arabidopsis thaliana* the apical meristem generates primordia on its flanks in a phyllotactic spiral, and, as development progresses, these primordia are specified as rosette leaves with axillary shoot meristems, cauline leaves with similar meristems, or flowers (Smyth et al., 1990).

Some of the genes involved in primordium specification have been identified. Key factors specifying floral meristem identity include *LEAFY* (Schultz and Haughn, 1991; Weigel et al., 1992; Huala and Sussex, 1992; Weigel and Meyerowitz, 1993), *APETALA1* (Irish and Sussex, 1990; Mandel et al., 1992; Bowman et al., 1993) and *CAULI-FLOWER* (Bowman et al., 1993). *LEAFY* and *APETALA1* have been cloned, and their RNA is present in nascent and developing floral primordia (Weigel et al., 1992; Mandel et al., 1992).

Less is known about the other process, namely how the apical meristem structure is established and maintained. In classical botany, the apical meristem is divided into two regions, a central zone (CZ) and a peripheral zone (PZ) (Vaughn, 1952; Vaughn and Jones, 1953; Brown et al., 1964). Under this model, organ primordium initiation occurs in the PZ, while the CZ consists of slowly dividing cells that replenish the PZ. The apical meristem is further organized into three cell layers that in general remain clonally distinct throughout development (Satina et al., 1940; Miksche and Brown, 1965). The study of fasciation, where the meristem grows abnormally as a band- or ring-like structure, has fascinated naturalists for centuries (Linneaus, 1751; Moquin-Tandon, 1840; Masters, 1869; White, 1916, 1948; Gorter, 1965). Fasciated plants have even been the object of worship (Tadulingam and Jacob, 1925). Fasciation has often been found to be due to a single, recessive trait. Examples of this have been found in pea (Mendel, 1866), *Celosia cristata* (White, 1948), tobacco (White, 1916), tomato (Young and MacArthur, 1947; Mertens and Burdick, 1954; Szymkowiak and Sussex, 1992) *Zea mays* (Emerson, 1911) and soybean (Takagi, 1929). More recently, Leyser and Furner (1992) have shown that *Arabidopsis* plants with mutations in *CLAVATA1* (*CLV1*), *FASCIATA1* or *FASCIATA2* can exhibit disrupted phyllotaxy, inflorescence fasciation, and altered leaf development. They further demonstrated that 5 day old *clv1* apical meristems are taller and wider than wild type, while 5 day old *fas1* apical meristems are wider but shorter than wild type. A preliminary investigation has identified three mutations, *fey*, *dip* and *shz*, that affect apical meristem structure (Medford et al., 1992), but these mutations can also disrupt cotyledon and leaf development, and cause meristem necrosis, so these genes may not be specific to meristem development. Medford et al. (1992) also presented an initial analysis of *fuf*, a mutation that causes inflorescence fasciation, and demonstrated that the *fuf* apical meristem is enlarged at 7 days old compared to wild type.

The same two processes mentioned above as necessary for apical meristem development are also involved in flower development: the establishment of floral meristem structure, and the determination of floral organ identity. Our understanding of floral organ specification has been advanced by the characterization and cloning of the homeotic gene loci *AGAMOUS*, *APETALA3* and *PISTILLATA* (Bowman et al., 1991; Yanofsky et al., 1990; Drews et al., 1991; Jack et al., 1992; K. Goto and E. M. M., unpublished data), and the characterization of *APETALA2* (Bowman et al., 1991). Very little is known, on the other hand, about how floral organ number and position are determined. Smyth et al. (1990) described wild-type organogenesis in developing *Arabidopsis* flowers. Flowers initially arise as undifferentiated bulges on the flanks of the apical meristem and soon give rise to four sepal primordia establishing the first whorl (stage 3 - all stages according to Smyth et al., 1990). After the sepals grow to overlie the floral primordium (stage 4), four petal and six stamen primordia arise (stage 5), and soon after, carpel primordia (stage 6) initiate in the center of the floral meristem. Each distinct organ type occupies a separate whorl, with sepals in the first whorl, petals in the second whorl, stamens in the third whorl, and carpels in the fourth whorl. Leyser and Furner (1992) described three *clv1* alleles that exhibited a variable number of extra organs in each whorl.

Here we present a morphological and genetic analysis of a series of mutations in the *Arabidopsis CLAVATA1* locus. Our investigations have revealed that *clv1* disrupts the apical meristem structure by enlarging it in weak alleles, causing fasciation in intermediate alleles and causing massive overproliferation in the strongest allele. We have also observed increases in the size of *clv1* floral primordia at the earliest stages of organ initiation, and that the degree of increase in floral meristem size correlates with the increase in floral organ number in the mutant alleles. We report here that *clv1* further disrupts the floral meristem structure by developing additional whorls of carpels inside of the gynoecium generated by the fourth-whorl carpels. Genetic interactions between *clv1* mutations and mutations of six other genes have been investigated. We observe additive phenotypes for *clv1* double mutants with *ap2*, *ap3*, *pi* and *ag*, indicating *clv1* affects the underlying floral meristem structure upon which floral homeotic genes act. We demonstrate that *clv1* further affects floral meristem structure by altering patterns of *AGAMOUS* and *APETALA1* expression compared to wild type. Mutant combinations with *ap1* result in the transformation of the center of the flower into meristem tissue, and mutant combinations with *lfy* result in almost complete transformation of the flowers into shoot structures, indicating that *CLV1* plays a hitherto unsuspected role in floral meristem identity. We discuss models for the generation of fasciation and possible mechanisms of action for *CLV1* in *Arabidopsis* development.

MATERIALS AND METHODS

For all plants, seeds were sown on a thin covering (5-10 mm) of Redi-earth peat-lite mix over a 1:1:1 mixture of perlite:vermiculite:soil. After 4 days at 4°C, plants were placed under lights. All plants, except *ag-2* and *clv1-1 ag-2*, were grown under 600 ft-candles of constant cool white fluorescent light at 23-25°C. Plants were fertilized at about 10 days after germination. *ag-2* flowers grown under the above conditions often lack sepals in the inner whorls (whorls 4, 7, 10, etc.), making organ counts as in Table 3 difficult. Therefore, *ag-2* and *clv1-1 ag-2* plants were grown in a Percival chamber under constant 225 ft-candles of cool white fluorescent at 25°C. These conditions are identical to those in Bowman et al., 1989, 1991. Short day conditions were also carried out in a Percival chamber. Plants were subjected to a 10 hour light/ 14 hour dark cycle under 325 ft-candles of cool white fluorescent light at 24°C. Various *clv1* phenotypes, especially fasciation, are sensitive to general growing conditions and crowding in particular. Thus seeds were sown at a maximum density of 1 seed per 4 cm^2.

clv1-1 is an ethyl methanesulfonate (EMS)-induced allele originally isolated in ecotype Landsberg by Koornneef et al. (1983) (see Table 1). *clv1-4* is an X-ray induced allele isolated in either Estland or Limburg ecotype by McKelvie (1962). For double mutant combination with *lfy-1* (Fig. 12) and for SEM analysis in Fig. 1D-F, *clv1-4* was first backcrossed to ecotype Columbia. For all other analyses, *clv1-4* was backcrossed to ecotype Landsberg *erecta* three times. *clv1-5* appeared spontaneously in plants of Landsberg *erecta* background. *clv1-5* was backcrossed to ecotype Landsberg *erecta* twice.

A specific screen was performed to isolate additional *clv1* alleles. To do so, *clv1-1 pi-1* double mutant plants in Landsberg were hand pollinated with ecotype Columbia pollen that had been subjected to 7500 rads of X-rays. The 15,000 seeds that developed were grown and screened for phenotypically *clv1* plants. 5 phenotypically *clv1* plants were identified, and seeds from these selfed M_1 plants were grown to verify that no wild-type plants segregated out. Seed was collected from individual M_2 plants, and DNA was isolated from pools of M_3 plants, each pool representing a single M_2 plant. For each new *clv1* allele, M_3 DNA from 15-20 M_2 individuals was analyzed by Southern hybridization for restriction fragment length polymorphism (RFLPs). Cosmids N7-24, KG-24 and 532 map in the region of *clv1* (S. E. C., R. W. Williams and E. M. M., unpublished data), and were used as probes. For three of the new alleles, all M_2 plants were homozygous for Landsberg-specific RFLPs, indicating that the X-ray induced allele had been lost. This is possibly the result of a large deletion (perhaps a chromosome arm) that is unable to transmit through gametes. For two of the new alleles, Landsberg- and Columbia-specific RFLPs were found in an approximately 1:1 ratio, indicating that the new allele had transmitted to the next generation. For each of these new alleles (*clv1-6* and *clv1-7*), an M_2 plant homozygous for Columbia-specific RFLP was chosen and an M_3 offspring from it was back crossed to Landsberg *erecta* before analysis.

Scanning electron microscopy (SEM) was performed as described (Bowman et al., 1989).

In situ hybridizations were carried out according to Drews et al. (1991), using synthesized *AG* probe as described. *AP1* probe was synthesized from the pAM128 plasmid kindly supplied by A. Mandel and M. Yanofsky (Mandel et al., 1992).

For confocal laser scanning microscopy (CLSM) of flowers, the following protocol was developed. Inflorescence tissue was fixed for 4 days in a solution of 10:5:70 formalin:propionic acid:ethanol. The tissue was taken through an ethanol series to 100% ethanol and left in 100% ethanol overnight to eliminate chlorophyll. The tissue was then rehydrated and stained for 6 days in 0.1 M L-arginine, pH 12.4, with 5 mg/ml propidium iodide, and destained for 4 days in 0.1 M L-arginine, pH 8. After ethanol and xylene series, the tissue was cleared for several hours in 100% xylene. All series were performed gradually, using several steps each with small changes in ethanol and/or xylene concentration. The tissue was dissected and mounted in immersion oil, and viewed with an

argon laser (488 and 514 nm excitation wavelength) using the Zeiss confocal LSM 10 microscope. Images were cropped and the brightness and contrast were adjusted using Adobe Photoshop 2.0 for Macintosh computers, and printed using a Nikon printer at 200 dpi resolution.

RESULTS

clavata1 alleles

We have investigated five *clv1* alleles as listed in Table 1. *clv1-1* is the original *clv1* allele isolated by Koornneef et al. (1983). *clv1-4* was isolated by McKelvie (1962). *clv1-5* was a spontaneous mutation isolated in this lab. *clv1-6* and *clv1-7* were isolated in a pollen mutagenesis screen designed to identify new *clv1* alleles (see Materials and methods).

clavata1 mutants have abnormal apical meristems

It is clear from superficial examination that *clavata1* (*clv1*) mutants exhibit defects in the inflorescence meristem (IM). First, the primary IM is usually fasciated in the stronger alleles *clv1-4* and *clv1-1*. These fasciated meristems grow as a strap-like structure (Leyser and Furner, 1992). Additionally, the normal spiral phyllotaxis of emerging flowers (Smyth et al., 1990) is disrupted, suggesting that some aspect of floral primordium initiation on the flanks of the apical meristem is affected.

To determine the nature of these defects in greater detail, inflorescence meristems were fixed and examined by scanning electron microscopy (SEM). *clv1-5* plants exhibited disrupted phyllotaxy, but were only rarely fasciated. As shown in Fig. 1A,B, the apical meristem of *clv1-5* plants was enlarged in width and height compared to that of wild-type plants. The *clv1-5* apical meristem had a larger number of young floral primordia at its flanks, and these floral primordia did not appear to be spirally arranged. *clv1-1* and *clv1-4* apical meristems, on the other hand, developed as fasciated meristems when grown at 25°C. These fasciated meristems grew as a line instead of a point, with floral primordia developing along the sides of the apical meristem line (Fig. 1C-F). An even more dramatic defect in inflorescence meristem structure could be observed when *clv1-4* plants were grown at 16°C. The *clv1-4* meristem, instead of becoming fasciated, developed into a huge misshapen mass that dwarfed the apical meristem of wild-type plants grown at 16°C (Fig. 1G,H). Despite this disruption in the apical meristem, floral primordia were still generated at its base.

Table 1. *clavata1* alleles

Allele	Mutagen	Source
clv1-1	EMS	Koornneef (1983)
clv1-4	X-ray	McKelvie (1962)
clv1-5	unknown	This paper
clv1-6	X-ray	This paper
clv1-7	X-ray	This paper

clavata1 alleles analyzed in this study are listed. See Materials and methods for description of *clv1-5*, *clv1-6* and *clv1-7* isolation. EMS, ethyl methanesulfonate.
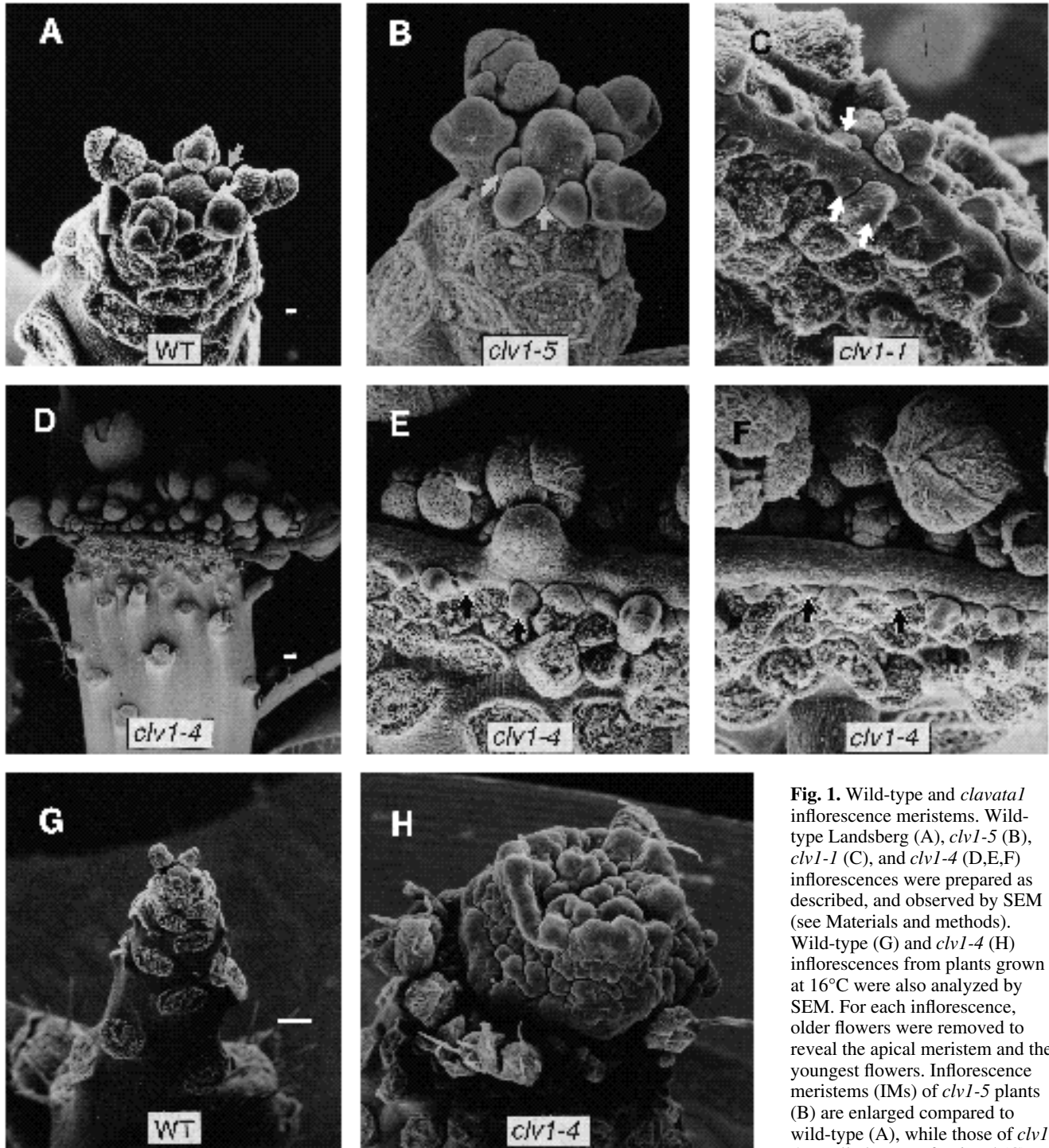
To determine how a fasciated meristem is generated, *clv1-5*, *clv1-1*, *clv1-4* and wild-type plants grown at 25°C were examined at day 8 through day 13 of development (with day 1 being 24 hours after removal from cold shock, see Materials and methods). By day 7, the wild-type vegetative meristem has generated 5 leaf primordia (Medford et al., 1992). In our growth conditions, the period from day 8 through 13 includes the development of the last rosette leaves, the cauline leaves and the initial flower primordia, and thus includes the transition to flowering. As Fig. 2 shows, the apical meristem of *clv1* plants was both wider and taller than wild-type plants at day 8, with the increase in *clv1* meristem size depending on the severity of the allele. The *clv1-1* meristem continued to grow larger until about 11-12 days, during the transition from vegetative to floral growth, when the *clv1-1* apical meristem extended out in one or both directions, leading to fasciation (Fig. 2K,O). These extensions occurred at the shoulders of the apical meristem at the approximate locations where leaf and flower primordia are generated. The prominence of the original apical meristem was sometimes retained (Fig. 1E) and sometimes lost (data not shown) as flowering progressed. *clv1-5* apical meristems, on the other hand, retained their radial symmetry, and thus rarely became fasciated (Fig. 2J,N). *clv1-4* exhibited the greatest increase in apical meristem size as compared to wild type, forming apical meristem structures with a variety of shapes (Fig. 2).

Because the transition to flowering and the initial occurrence of fasciation coincide during development, we sought to determine if the transition to flowering was necessary for fasciation. Wild-type, *clv1-1*, and *clv1-4* plants were grown under short day conditions (10 hours light/14 hours dark), in which the transition to flowering is delayed, and, as a result, the vegetative growth phase is extended. Unexpectedly, *clv1-4* plants were not affected by the short day light regime and bolted after as few as 26 days. Wild-type and *clv1-1* plants, on the other hand, did not bolt until 55 and 60 days, respectively. Fig. 3 shows apical meristems from these plants at 31 days. The *clv1-1* and wild-type meristems were still generating leaves (Fig. 3A,B), while the *clv1-4* plant had a well-developed inflorescence meristem with mature flowers (Fig. 3C). Despite the fact that *clv1-1* plants had not undergone a transition to flowering, the apical meristems of these plants were clearly fasciated, indicating that the floral transition is not necessary for fasciation.

clavata1 mutants have increased floral organ number

Plants mutant for *clv1* were initially identified as having an increase in carpel number (Koornneef et al., 1983), but subsequent analysis revealed that the mutation causes an increase in the number of all floral organ types (Leyser and Furner, 1992). We analyzed five *clv1* alleles for the number of organs in each whorl (see Table 1 and Materials and methods for origin of alleles). Fig. 4 shows a flower from a weak (*clv1-6*, Fig. 4B) and strong (*clv1-4*, Fig. 4C) allele in comparison with a wild-type flower (Fig. 4A). Fig. 5 shows mean floral organ numbers for each mutant allele. *clv1-4* plants exhibited the greatest increase in organ number. The severity of the phenotype of other *clv1* alleles was in decreasing order: *clv1-1* > *clv1-5* > *clv1-6* > *clv1-7*.

Fig. 1. Wild-type and *clavata1* inflorescence meristems. Wild-type Landsberg (A), *clv1-5* (B), *clv1-1* (C), and *clv1-4* (D,E,F) inflorescences were prepared as described, and observed by SEM (see Materials and methods). Wild-type (G) and *clv1-4* (H) inflorescences from plants grown at 16°C were also analyzed by SEM. For each inflorescence, older flowers were removed to reveal the apical meristem and the youngest flowers. Inflorescence meristems (IMs) of *clv1-5* plants (B) are enlarged compared to wild-type (A), while those of *clv1-1* usually become fasciate (C). *clv1-4* IMs fasciate in an *ER* background (D - closer view in E and F), and often the original apical meristem is still distinct (E). Note parallel rows of cells on the fasciated meristem in (F). When grown at 16°C, *clv1-4 er* IMs (H) become massively proliferative and dwarf wild-type IMs grown at 16°C (G). Floral primordia are indicated with arrows. Bars, 10 μm (A) and 100 μm (D,G). Note A-C,E and F are shown at identical magnification, as are G and H. All plants except D,E and F carried the *erecta* mutation.

Except for the weakest alleles, *clv1-6* and *clv1-7*, the mean numbers of organs in all four whorls were increased in homozygous mutant plants. Organ numbers in the inner two whorls (carpels and stamens) were more affected than organ numbers in the outer two whorls (sepals and petals) in all

alleles. *clv1-4* and *clv1-1* also showed a semi-dominant phenotype. The early flowers that developed on *clv1-1/+* and *clv1-4/+* plants had extra fourth-whorl carpels, but they did not have a detectable fifth whorl (data not shown), as do homozygous *clv1* flowers (see below). No other
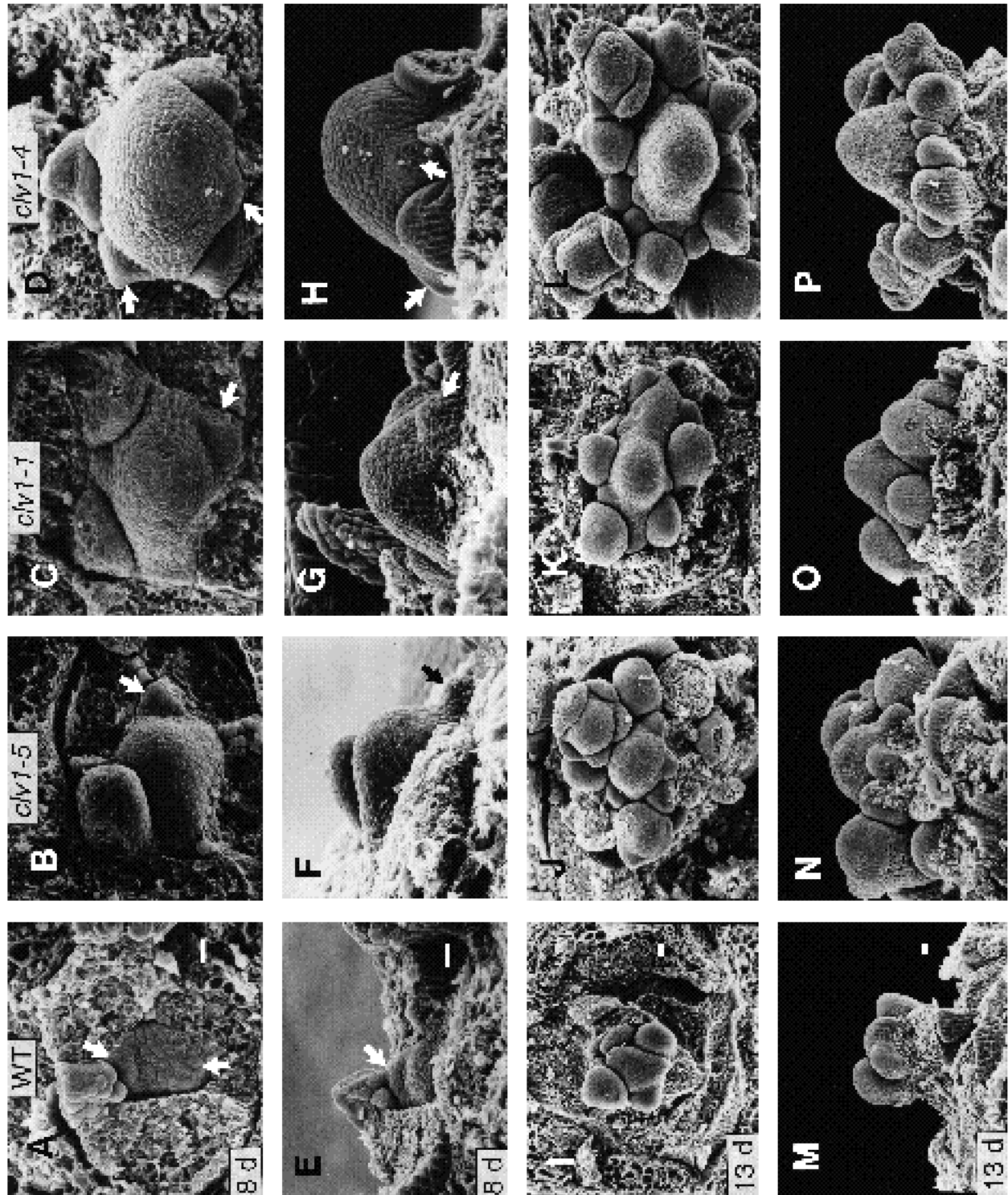
Fig. 2. Apical meristems of wild-type and *clavata1* plants at day 8 and 13. Landsberg (A,E,I,M), *clv1-5* (B,F,J,N), *clv1-1* (C,G,K,O) and *clv1-4* (D,H,L,P) plants were prepared as described and examined by SEM. Plants grown for 8 days are shown in A-H, and those grown for 13 days are shown in I-P. E-H and M-P are side views of apical meristems shown in A-D and I-L, respectively. A-H are shown at the same magnification, as are I-O. Arrows indicate leaf primordia. All plants carried the *erecta* mutation. Bar, 10 μm.
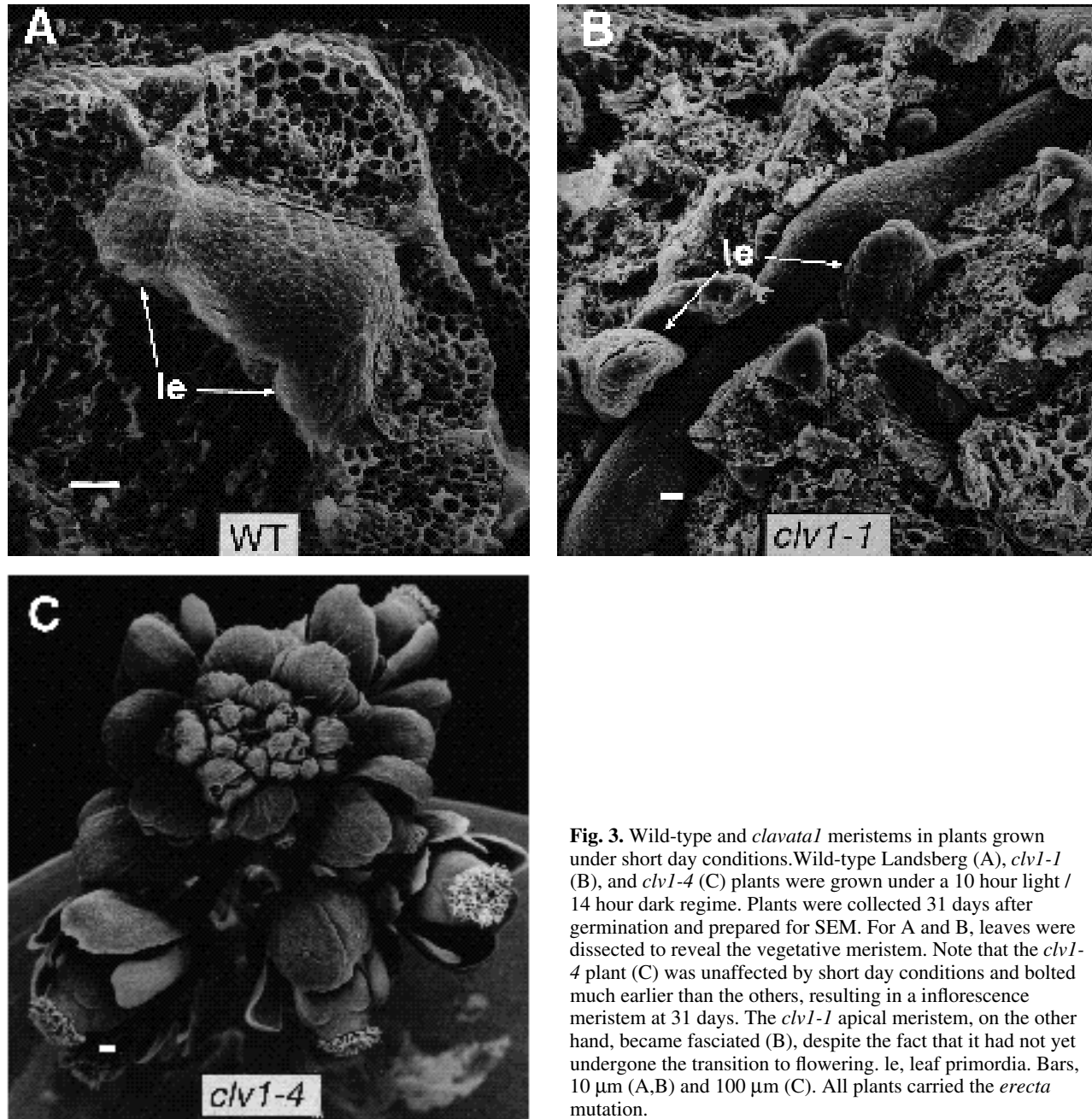
Fig. 3. Wild-type and *clavata1* meristems in plants grown under short day conditions.Wild-type Landsberg (A), *clv1-1* (B), and *clv1-4* (C) plants were grown under a 10 hour light / 14 hour dark regime. Plants were collected 31 days after germination and prepared for SEM. For A and B, leaves were dissected to reveal the vegetative meristem. Note that the *clv1-4* plant (C) was unaffected by short day conditions and bolted much earlier than the others, resulting in a inflorescence meristem at 31 days. The *clv1-1* apical meristem, on the other hand, became fasciated (B), despite the fact that it had not yet undergone the transition to flowering. le, leaf primordia. Bars, 10 μm (A,B) and 100 μm (C). All plants carried the *erecta* mutation.

consistent defects were observed in *clv1-1*/+ and *clv1-4*/+ plants.

clavata1 mutants have increased floral meristem size and whorl number

To determine the effect of *clv1* mutations on early stages of flower development, wild-type and *clv1-1* flowers were analyzed by confocal laser scanning microscopy (CLSM). CLSM has the advantage over conventional sectioning techniques in that it can be used to rapidly take sections through whole-mount tissue by optically filtering background fluorescence above and below the plane of focus. Its use in plants had been limited to studies of autofluorescent tissue such as pollen grains and ovules, presumably because of the difficulty that the cuticle and cell wall present to the staining

of whole-mount plant tissue. We have developed a protocol for fixing and clearing plant tissue that has been stained with propidium iodide, a nuclear stain, which allows CLSM analysis (see Materials and methods). As shown in Fig. 6, optical sections through stage 3 flowers (all stages according to Smyth et al., 1990) revealed that *clv1* floral meristems were taller than wild-type meristems (Fig. 6A-C). The floral meristem size from the inner base of the sepal primordia to the top of the flower was measured for CLSM sections and paraffin sections of stage 3 wild-type *clv1-1* and *clv1-4* flowers (Table 2). The mean floral meristem height of 12 μm in wild-type flowers was dramatically increased to 23 μm in *clv1-1* flowers and 41 μm in *clv1-4* flowers. There was a less dramatic increase in the width of *clv1* stage 3 flowers. The mean width of the floral primordia between the
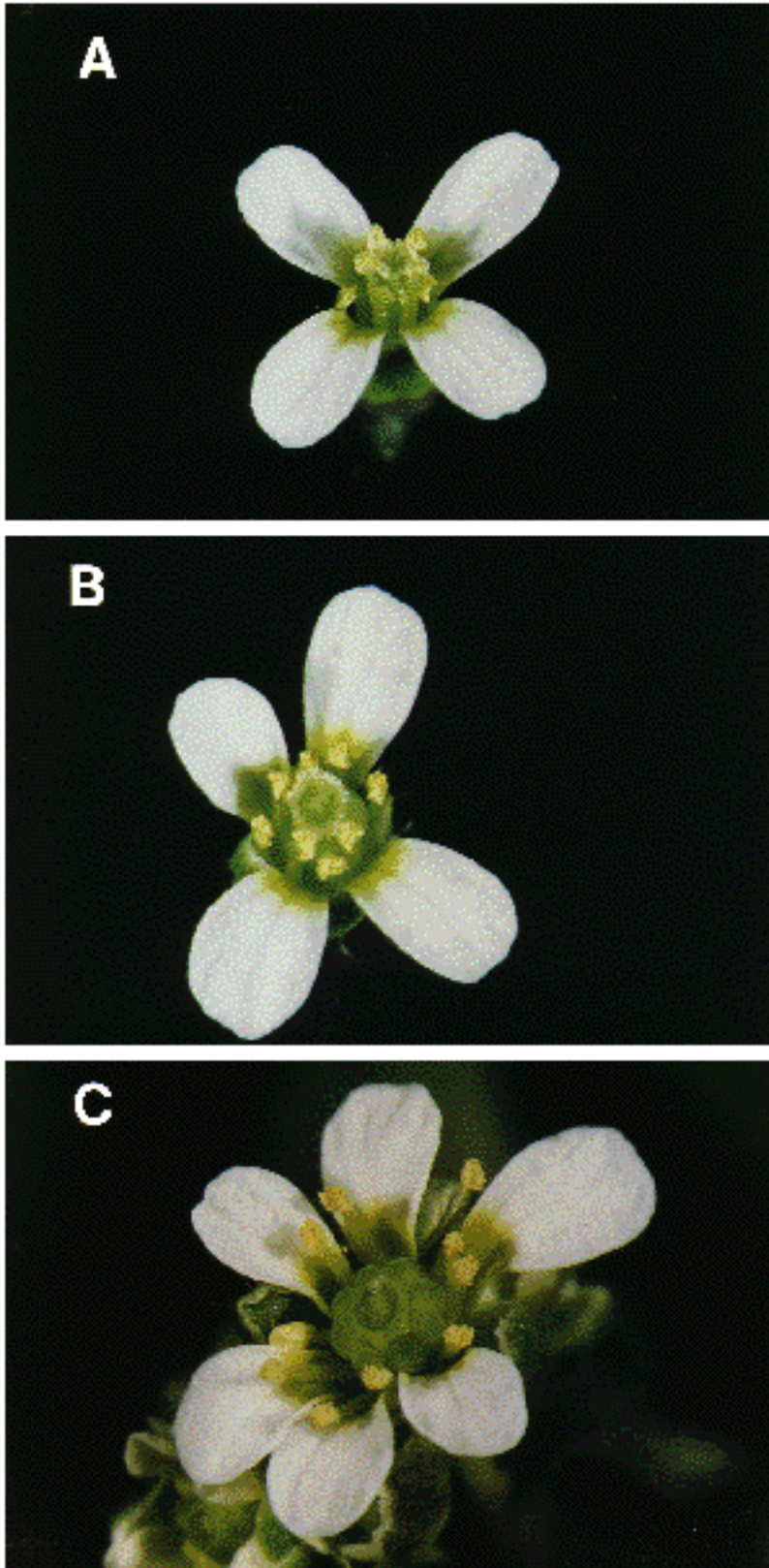
Fig. 4. Wild-type and *clavata1* flowers. Shown are photos of wild-type Landsberg (A), a weak *clavata1* allele, *clv1-6* (B), and a strong *clavata1* allele, *clv1-4* (C). All plants carried the *erecta* mutation.

inner bases of the sepal primordia were 49 μm for wild type, 55 μm for *clv1-1* and 66 μm for *clv1-4*.

To determine if this increase in floral meristem size in *clv1* flowers was due to more or larger cells, nuclei were counted in the CLSM sections shown in Fig. 6. The wild-type flower contained 21 nuclei in the region measured above, while the *clv1-1* flower contained 61 nuclei and the *clv1-4* flower contained 73 nuclei.
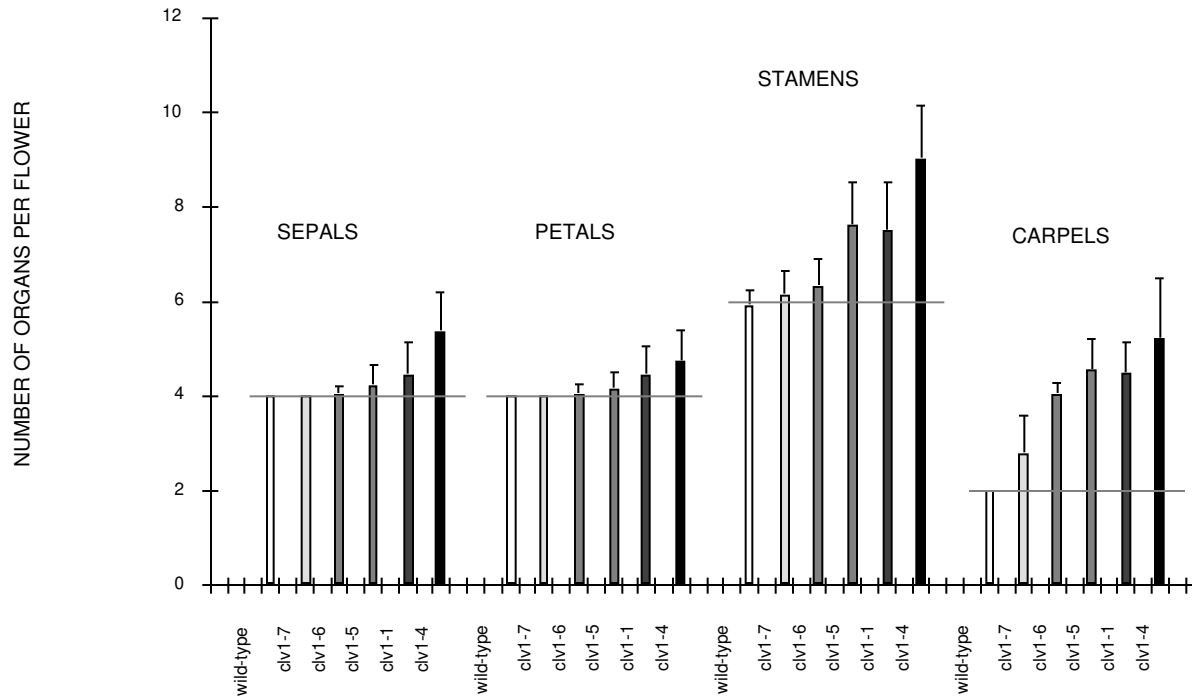
Fig. 5. The number of organs in wild-type and *clavata1* flowers. *Bars* represent the mean number of indicated organs for flowers of wild-type Landsberg, *clv1-7*, *clv1-6*, *clv1-5*, *clv1-1* and *clv1-4* plants. At least 90 flowers were counted for each mean calculated. Only the first ten flowers on any given plant were analyzed. Standard errors are indicated by vertical *black lines*. The dotted horizontal lines represent the normal wild-type organ number. *clv1-5* stamens include 0.39 antherless filaments per flower. *clv1-1* stamens include 0.32 antherless filaments per flower. *clv1-4* stamens include 0.52 antherless filaments per flower. Note that the upper limit of the mean plus standard error for the number of stamens for wild-type flowers exceeds six due to flowers with 4 and 5 stamens, but no wild-type flowers were observed with more than six stamens. All plants carried the *erecta* mutation.

Table 2. Floral meristem sizes at stage 3

Genotype	Width (μm)	Height (μm)	n
WT	49 ± 6	12 ± 3	23
clv1-1	55 ± 5	23 ± 6	21
clv1-4	66 ± 9	41 ± 7	13

Confocal laser scanning microscopy sections (see Materials and methods) and 6 μm paraffin sections of Landsberg, *clv1-1* and *clv1-4* stage 3 flowers were measured. Width measurements were taken between the base of the sepal primordia. Height measurements were taken from the inner base of the sepal primordia to the top of the flower. The mean width and height of wild-type, *clv1-1* and *clv1-4* flowers are presented and the standard error for each mean is indicated. The number of flowers measured for each mean is listed under *n*. All plants carried the *erecta* mutation.

At stage 6 in wild-type flowers, carpel primordia arose at the center of the floral meristem, at which time the floral meristem terminates (Fig. 6D), while in *clv1-1* flowers the fourth-whorl carpels arose on the flanks of a central dome (Fig. 6E). Optical sections of *clv1-4* flowers showed that this central dome was enlarged compared to *clv1-1* flowers (Fig. 6F). SEM of stage 6 *clv1-1* and *clv1-4* flowers also indicated that the carpels arose as a ring of organs around a still-proliferating central dome, with the dome in *clv1-4* flowers being larger (Fig. 6G-I). We define this central dome as a fifth whorl.

To determine the fate of this fifth whorl, *clv1-1* gynoecia

were examined late in development after the outer organs had senesced and as seeds were developing (stage 17). After removing the outer silique coat, we observed an additional gynoecium inside of the gynoecium generated by the fourth-whorl carpels (Fig. 7A). This inner gynoecium was found in every *clv1-1* silique in over 100 flowers analyzed. In weaker alleles, this inner gynoecium was much reduced in size (Fig. 7B), while in the strongest allele, *clv1-4*, the growth of this inner gynoecium was dramatically increased. In a typical *clv1-4* gynoecium, as shown in Fig. 7C, a 'sixth whorl' gynoecium was observed inside of the fifth-whorl gynoecium, with what appeared to be meristem tissue emerging out of this sixth whorl gynoecium (Fig 7D, cf. Figs 1H, 9H, 12G and 14B). In rare cases, a new inflorescence with developing flowers emerged from the gynoecium in *clv1-4* flowers (data not shown).

Genetic combinations with other flower mutations

Extensive genetic studies have examined the role of the homeotic loci *APETALA2*, *APETALA3*, *PISTILLATA*, and *AGAMOUS* in establishing floral organ identity (Bowman et al., 1989; Meyerowitz et al., 1991; Bowman et al., 1991; Coen and Meyerowitz, 1991). Double mutant combinations of *clv1-1* with the mutations *apetala2-9*, *apetala3-1* and *pistillata-1* were all additive in phenotype (data not shown). The addition of the *clv1-1* mutation resulted in extra organs of the type specified by the particular combinations of active homeotic genes (Bowman et al., 1989, 1991). As in *clv1-1*
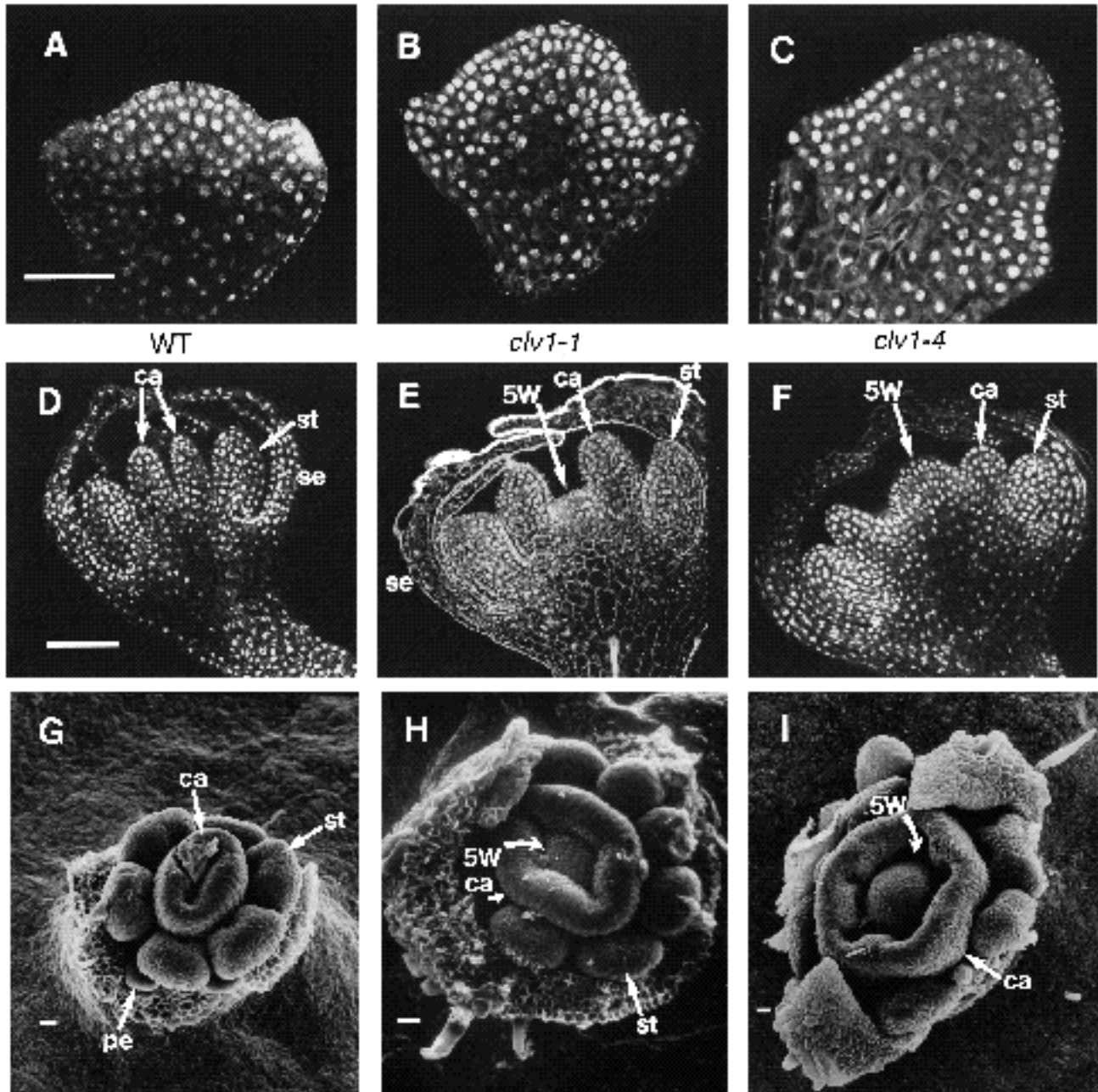
Fig. 6. Early development of wild-type and *clavata1* flowers. Wild-type Landsberg (A,D), *clv1-1* (B,E), and *clv1-4* (C,F) flowers were prepared for confocal laser scanning microscopy as described (see Materials and methods). (A-C) Optical sections were taken through stage 3 flowers. Note the taller floral meristem in *clv1-1* (B) and *clv1-4* (C) floral primordia. (D-F) Optical sections were taken through stage 6 flowers. Note that carpels in *clv1-1* and *clv1-4* flowers form on the flanks of the floral meristem instead of on the terminus as in wild type. Wild-type Landsberg (G), *clv1-1* (H), and *clv1-4* (I) flowers were prepared as described and observed by SEM after several sepals had been removed. Note the fifth whorl in *clv1-1* and *clv1-4* flowers. All plants carried the *erecta* mutation. se, sepal primordia; pe, petal primordia; st, stamen primordia; ca, carpel primordia; 5W, fifth whorl. Bars, 25 µm (A), 50 µm (D), 10 µm (G-I). Note A,B and C are shown at the same magnification, as are D,E and F.

alone, the effects on organ number were weaker in the outer whorls and stronger in the inner whorls.

The *AGAMOUS* (*AG*) locus controls both the organ identity of stamens and carpels and the determinate growth of the flower, such that plants mutant in *AG* produce flowers with an indeterminate (sepals, petals, petals)$_n$ phenotype

(Bowman et al., 1989). The phenotype of *clv1-1 agamous-2* doubly mutant plants was also essentially additive. As Table 3 shows, the number of organs in each whorl was increased in *clv1-1ag-2* flowers as compared to *ag-2* single mutant flowers. Fig. 8 shows a comparison between *ag-2* and *clv1-1 ag-2* floral meristems. While the floral meristem
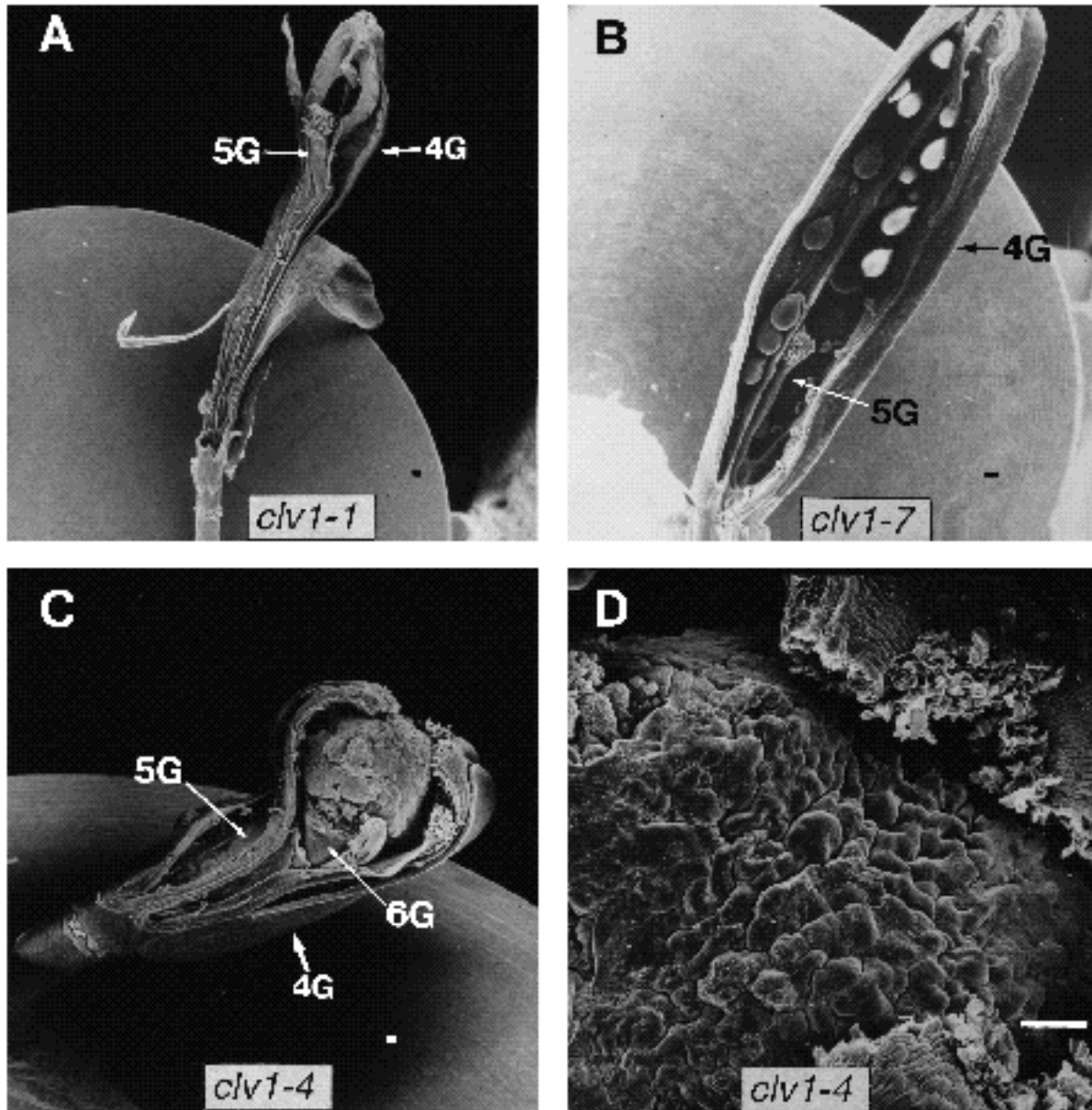
Fig. 7. The fate of the fifth whorl in *clv1* flowers. *clv1-1* (A), *clv1-7* (B), and *clv1-4* (C,D) stage 17 gynoecia were prepared as described, and observed by SEM after the facing silique coat had been removed to reveal the inner gynoecium. The fifth-whorl gynoecium is reduced in size in *clv1-7* (B), and greatly enlarged in *clv1-4* (C). (D) A top view of the *clv1-4* gynoecium in C. Note its similarity to meristem tissue in Figs 1H, 9H, 12G and 14B. 4G, gynoecium from fourth-whorl carpels; 5G, fifth-whorl gynoecium; 6G, sixth-whorl gynoecium. Bar, 100 μm. All plants carried the *erecta* mutation.

size was maintained throughout *ag-2* flower development (Fig. 8A), *clv1-1 ag-2* floral meristems became extremely fasciated (Fig. 8B-D).

The *APETALA1* (*AP1*) locus is involved in both floral organ identity and floral meristem identity (Irish and Sussex, 1990; Mandel et al., 1992; Bowman et al., 1993). *ap1-1* flowers consist of leaf-like sepals, few or no petals, and largely normal stamens and carpels (Fig. 9A,B). Also, additional flowers form in the axils of the leaf-like sepals (Irish and Sussex, 1990; Fig. 9B). *clv1-1 ap1-1* and *clv1-4 ap1-1* double mutant flowers displayed a surprising phenotype. Occasionally growing out of the center of the flower was a

new inflorescence meristem (Fig. 9C,E,G). In *clv1-1 ap1-1* flowers, the fourth-whorl gynoecium formed normally, and the new inflorescence grew through it (data not shown). Sections through developing *clv1-1 ap1-1* flowers indicated that the fifth whorl developed as an inflorescence meristem (data not shown). In *clv1-4 ap1-1* flowers with new inflorescences in the center of the flower, the fourth-whorl gynoecium usually did not form properly, and the carpels were often leaf-like, staminoid, or free standing (Fig. 9C,E). The frequency of new inflorescences in the center of *clv1-1 ap1-1* and *clv1-4 ap1-1* flowers was quite variable and higher in the early flowers. More common in *clv1-4 ap1-1*
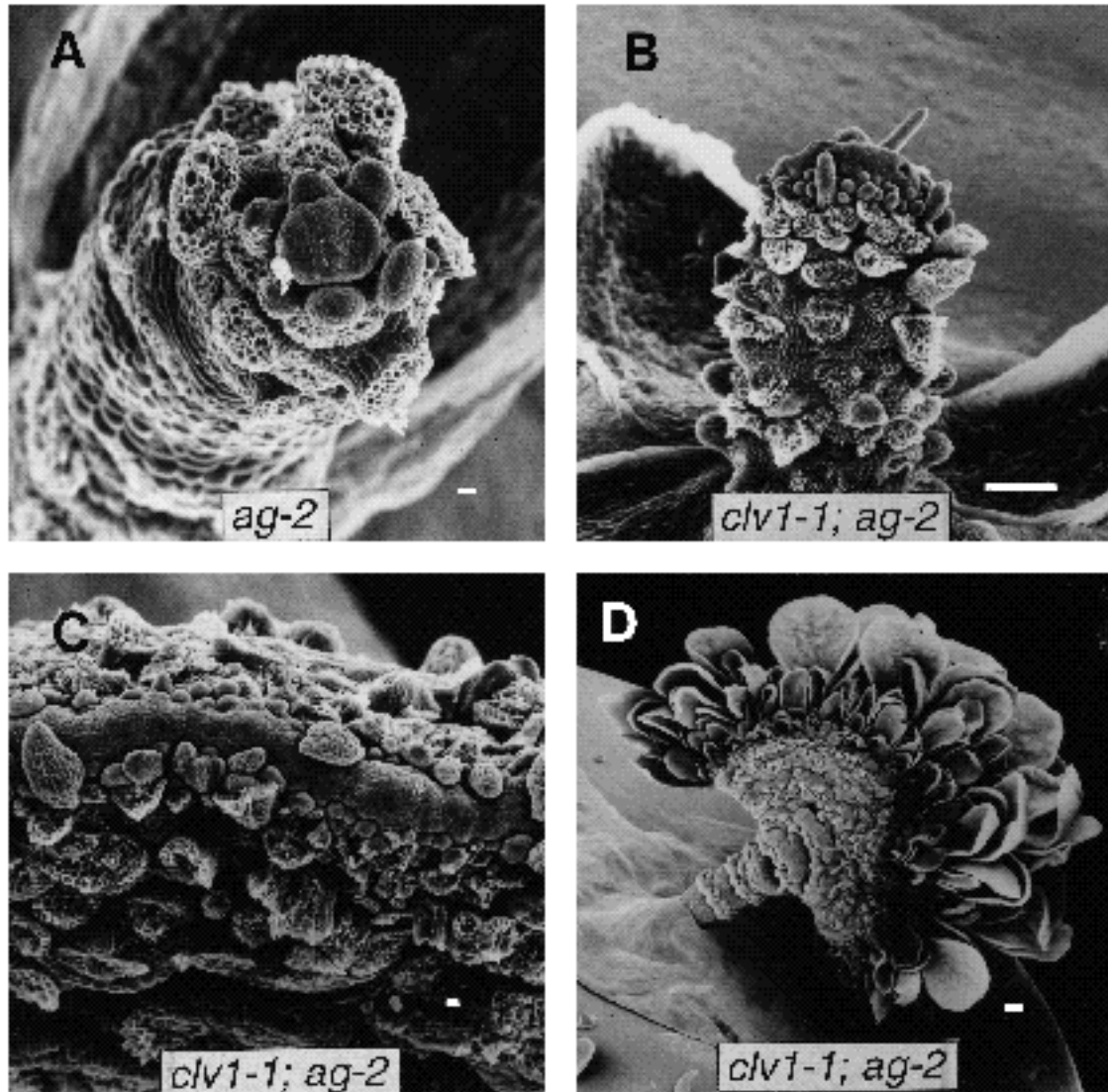
Fig. 8. *agamous* and *clavata1 agamous* flowers. *ag-2* (A) and *clv1-1 ag-2* (B-D) mature flowers were prepared as described and observed by SEM. An *ag-2* flower (A), and a *clv1-1 ag-2* flower (B) from which most organs have been removed are shown. Note the *clv1-1ag-2* flower has started to fasciate. (C) A portion of a fasciated floral meristem of an older *clv1-1 ag-2* flower from which most organs have been removed. (D) An older *clv1-1 ag-2* flower, from which organs on one side have been removed, displaying progressively increasing fasciation. Bars, 10 μm (A,C) and 100 μm (B,D). All plants carried the *erecta* mutation.

Table 3. Floral organs counts for *ag-2* and *clv1-1 ag-2*

Whorl	Organ type	*ag-2*	*clv1-1 ag-2*
Whorl 1	Sepals	4.0 ± 0	4.1 ± 0.4
Whorl 2+3	Petals	9.8 ± 0.5	11.7 ± 0.9
Whorl 4	Sepals	3.8 ± 0.4	4.7 ± 0.7
Whorl 5+6	Petals	8.3 ± 1.6	13.0 ± 1.1
Whorl 7	Sepals	2.2 ± 0.6	5.5 ± 0.8

The number of floral organs in each whorl of 40 *ag-2* and *clv1-1 ag-2* flowers were counted. Adjacent petal whorls were combined due to the difficulty in distinguishing between the two whorls. The mean number of indicated organs is shown with the standard error. Filaments were occasionally observed in the petal whorls: whorl 2 and 3 organ numbers include 0.08 filaments/flower for *ag-2* and 1.3 filaments/flower for *clv1-1 ag-2*; whorl 5 and 6 organ numbers include 0.03 filaments/flower for *ag-2* and 0.05 filaments/flower for *clv1-1 ag-2*. All plants carried the *erecta* mutation.

flowers was the phenotype shown in Fig. 9D,F,H. In these flowers the gynoecium fused normally, and a large growth burst through it (Fig. 9F,G). A close examination of this growth showed that it had a characteristic meristem appearance (Fig. 9H, cf. Figs 1H, 7D, 12G and 14B).

clavata1 mutants have altered patterns of *AGAMOUS* expression

AG controls the organ identity of the stamens and carpels, and consistent with its domain of function, *AG* is expressed in the developing third and fourth whorls (which give rise to stamens and carpels) beginning at stage 3 (Drews et al., 1991). Because *clv1* flowers are also somewhat indeterminate, in that they produce additional whorls compared to wild-type flowers, we were interested in examining the expression of *AG* in *clv1* flowers. Wild-type, *clv1-1* and
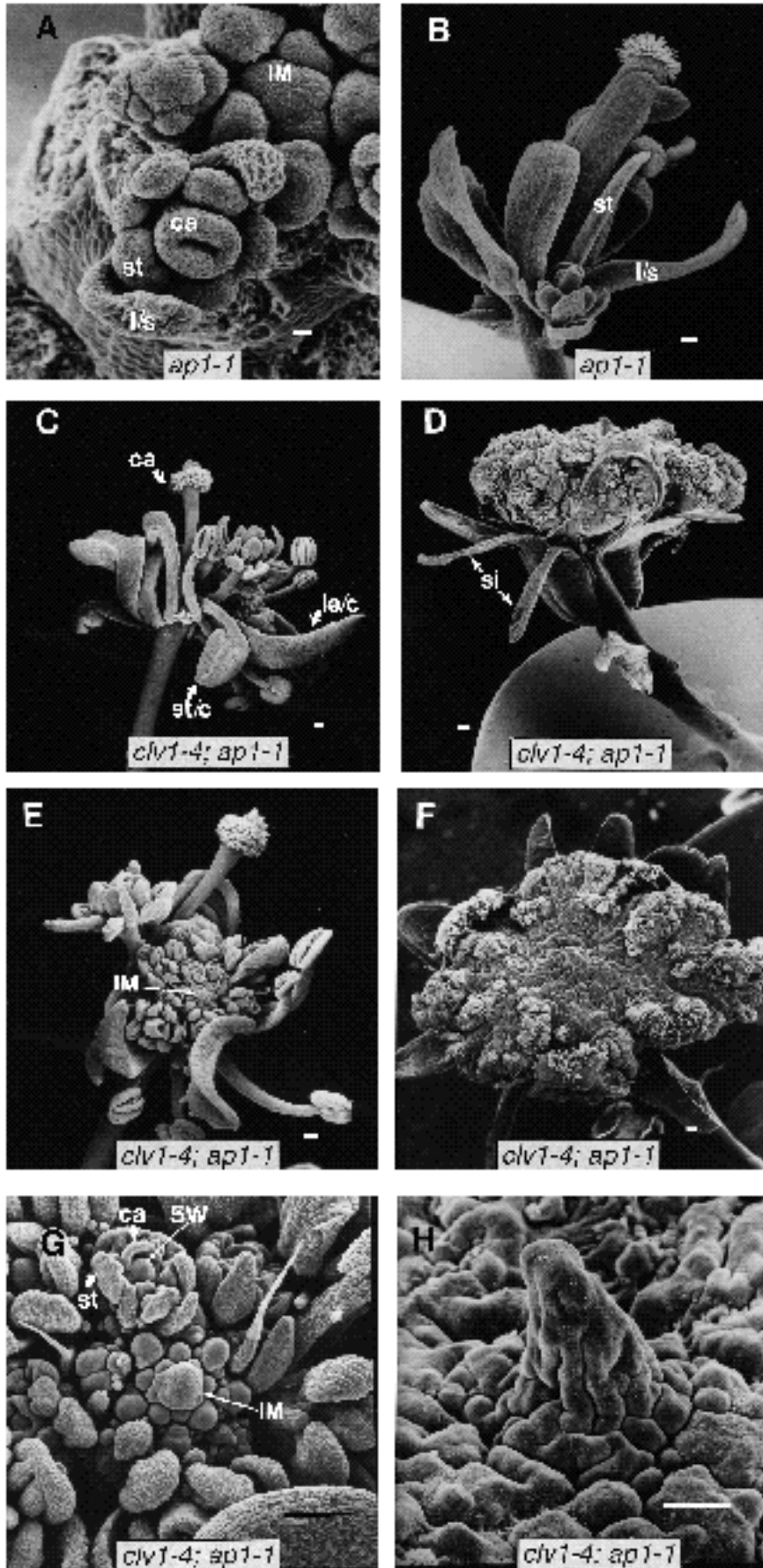
Fig. 9. *clavata1 apetala1* double mutant plants. *ap1-1* (A,B), and *clv1-4 ap1-1* (C-H) plants were prepared as described and examined by SEM. *ap1-1* inflorescence and stage 6 flower (A), and mature flower (B) are shown. *clv1-4 ap1-1* flowers with an inflorescence meristem (C,E,G) and meristem tissue (D,F,H) in the center of the flower are shown in side (C,D), top (E,F) and close-up (G,H) views. Note in C the staminoid, leaf-like or free-standing nature of the fourth-whorl carpels. G reveals that the center of the *clv1-4 ap1-1* flower (E) indeed contains an inflorescence meristem. This meristem has generated a number of young flowers including one in which the stamens (st), carpels (ca) and fifth whorl (5W) are indicated. Note in H the similarity to meristem tissue shown in Figs 1H, 7D, 12G and 14B. IM, inflorescence meristem; l/s, leaf-like sepal; st, stamen; ca, carpel; st/c, staminoid carpel; le/c, leaf-like carpel; si, silique coat; 5W, fifth whorl. Bars, 10 μm (A) and 100 μm for all other panels. All plants carried the *erecta* mutation.

clv1-4 flowers were fixed, sectioned and hybridized with a labeled *AG* probe (see Materials and methods). Fig. 10 shows the pattern of *AG* expression observed. In wild-type flowers, *AG* was expressed in the center of the flower in cells that will later develop into the stamens (whorl 3) and carpels (whorl 4; Fig. 10A,D,G). *clv1-1* and *clv1-4* flowers lacked *AG* expression in the center of the developing flower at the earliest stages of *AG* expression (Fig. 10B,C). *AG* was expressed in the portion of the *clv1* flower primordia that would have developed into the third and fourth-whorl organs, but not in the cells in the very center of the flower. In stage 6 *clv1-1* and *clv1-4* flowers, *AG* expression continued to be restricted to the stamens and fourth-whorl carpels, and was not present or was present at reduced levels in the fifth whorl (Fig. 10E,F). By stage 8, *AG* expression was detected in the fifth whorl of *clv1-1* flowers (Fig. 10H), but *clv1-4* flowers continued to lack *AG* expression at the tip of the still-proliferating floral meristem even at stage 12 (Fig. 10I,L), while stage 12 *clv1-1* flowers expressed AG throughout the gynoecium (Fig. 10J).

clavata1 mutants have altered patterns of *APETALA1* expression

The expression of *APETALA1* (*AP1*) is thought to be negatively regulated by *AG* (Mandel et al., 1992). *AP1* expression appears throughout stage 1 and 2 flowers, preceding the expression of the homeotic genes *AG*, *AP3* and *PI* (Mandel et al., 1992; Drews et al., 1991, Jack et al., 1992; K. Goto and E. M. M., unpublished data). This expression then becomes restricted to the first and second whorls, coinciding with the emergence of *AG* expression in the third and fourth whorls in stage 3 flowers.

Because *clv1* affects *AG* expression, and because *clv1 ap1* plants exhibit a non-additive phenotype, the expression of *AP1* was examined in *clv1* flowers. Sections of wild-type, *clv1-1* and *clv1-4* flowers were hybridized with a labeled *AP1* probe, and the results are shown in Fig. 11. At stages 1 and 2, expression in *clv1-1* and *clv1-4* flowers mirrored that in wild-type flowers (i.e. expression was observed in the entire flower primordium; Fig. 11A-C). As *AP1* expression in wild-type flowers is lost from the third and fourth whorls, *clv1* flowers displayed a retention of *AP1* expression in the center of the flower, but not in the cells that would have given rise to whorls 3 and 4. *AP1* expression in the fifth whorl of *clv1-1* flowers was low, but consistently observed in stage 6 flowers (Fig. 11E,G,H). This low level of expression persisted until about the stage that *AG* expression was observed in the fifth whorl (see Fig. 10H). In *clv1-4* flowers, the center of stage 3 flowers clearly retained *AP1* expression (Fig. 11F), but, *AP1* expression was not detected in the fifth whorl of stage 6 *clv1-4* flowers (Fig. 11I).

CLAVATA1 interacts with *LEAFY* to establish floral meristem identity

The *LEAFY* (*LFY*) locus is involved in establishing floral meristem identity (Schultz and Haughn, 1991; Weigel et al., 1992; Huala and Sussex, 1992). *lfy-1* plants produce extra cauline leaves before generating flowers. In addition, the flowers have some shoot meristem qualities: they are subtended by bracts, and the organs initiate in a slightly spiral pattern, rather than in perfect whorls (Weigel et al.,

1992; Fig. 12A). The flowers consist of sepals and poorly fused carpels. The double mutant combination of *clv1-1 lfy-1* was additive (Fig. 12B).

Unexpectedly, *clv1-4 lfy-1* double mutant plants displayed a more severe phenotype. After vegetative growth, the *clv1-4 lfy-1* plants generated only five to 15 'flowers', and produced hundreds of filamentous organs and bracts (Figs 13 and 12C). The flowers that were generated showed severe defects and could only loosely be characterized as flowers. First, all traces of a whorled pattern of organ initiation were absent (Fig. 12D). Second, the sepals were leaf-like: they were often flanked by stipules at their base, and they produced stellate trichomes (Fig. 12E). Third, internode elongation was often observed between the spirally initiated organs (Fig. 12E,F). Interior leaf-like organs were often tipped with stigmatic tissue, but lacked other carpel characteristics, such as ovules (Fig. 12E). Finally, the floral meristems were indeterminate and continued to generate leaf-like organs (Fig. 12E,F). In all, the 'flowers' had far more shoot characteristics than floral characteristics, demonstrating that *clv1-4 lfy-1* double mutant plants display a more severe phenotype than *lfy-1* single mutant plants in terms of the transformation of flowers into shoot structures. *clv1-4 lfy-1* plants demonstrate a clear disruption in floral meristem identity. The fact that *clv1-1 lfy-1* plants do not exhibit this defect suggests that *LFY-CLV1* interactions are sensitive to the level or quality of *CLV1* activity.

clv1-4 lfy-6 er plants were also analyzed (Note: *lfy-1* and *lfy-6* have identical lesions, CAG to TAG at codon 32). As shown in Fig. 12G, *lfy-6* enhanced the extent of meristem proliferation of *clv1-4* in an *er* background. In addition, secondary meristems became fasciated (Fig. 12H). The only other difference between *clv1-4 lfy-1* and *clv1-4 lfy-6 er* plants was that 'flowers' of the latter occasionally contained

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Fig. 10. *AGAMOUS* RNA expression in wild-type and *clavata1* plants. Landsberg (A,D,G), *clv1-1* (B,E,H,J) and *clv1-4* (C,F,I,K,L) inflorescences were fixed, sectioned and hybridized with labeled *AG* probe as described (see Materials and methods). All panels except K are bright-field/dark-field double exposures. Dark-field exposures were taken with a red filter to visualize the signal. In wild type, *AG* expression first appears in stage 3 flowers (A) in the center of the flowers in the anlage for stamens and carpels. *AG* expression continues to be present during stamen and carpel development (D,G). In *clv1-1* (B) and *clv1-4* (C) stage 3 flowers, *AG* expression is not present in the center of the flower, but is present in the region of whorls 3 and 4. As *clv1-1* flowers continue to develop, *AG* expression is lower or absent from the fifth whorl (E) until about stage 8 (H), where it begins to be clearly expressed in the fifth whorl. J shows a late *clv1-1* flower expressing *AG* throughout the gynoecium, including the fifth-whorl carpels. In contrast, as *clv1-4* flowers continue to develop, *AG* is never expressed in the very center of the flower. It is absent from the top of the fifth whorl in stage 6 and stage 8 flowers as shown (F,I). This proliferative dome eventually gives rise to fifth and sixth whorl carpels. K shows an approximately stage 12 flower, and *AG* expression in the proliferative dome is shown in L. Even at this late stage, *AG* is expressed at the base of this proliferative dome, but not at the tip. se, sepal; pe, petal; st, stamen; ca, carpel; 5W, fifth whorl; 4C, fourth-whorl carpel; 5C, fifth-whorl carpel. All plants carried the *erecta* mutation. Bars, 20 μm.
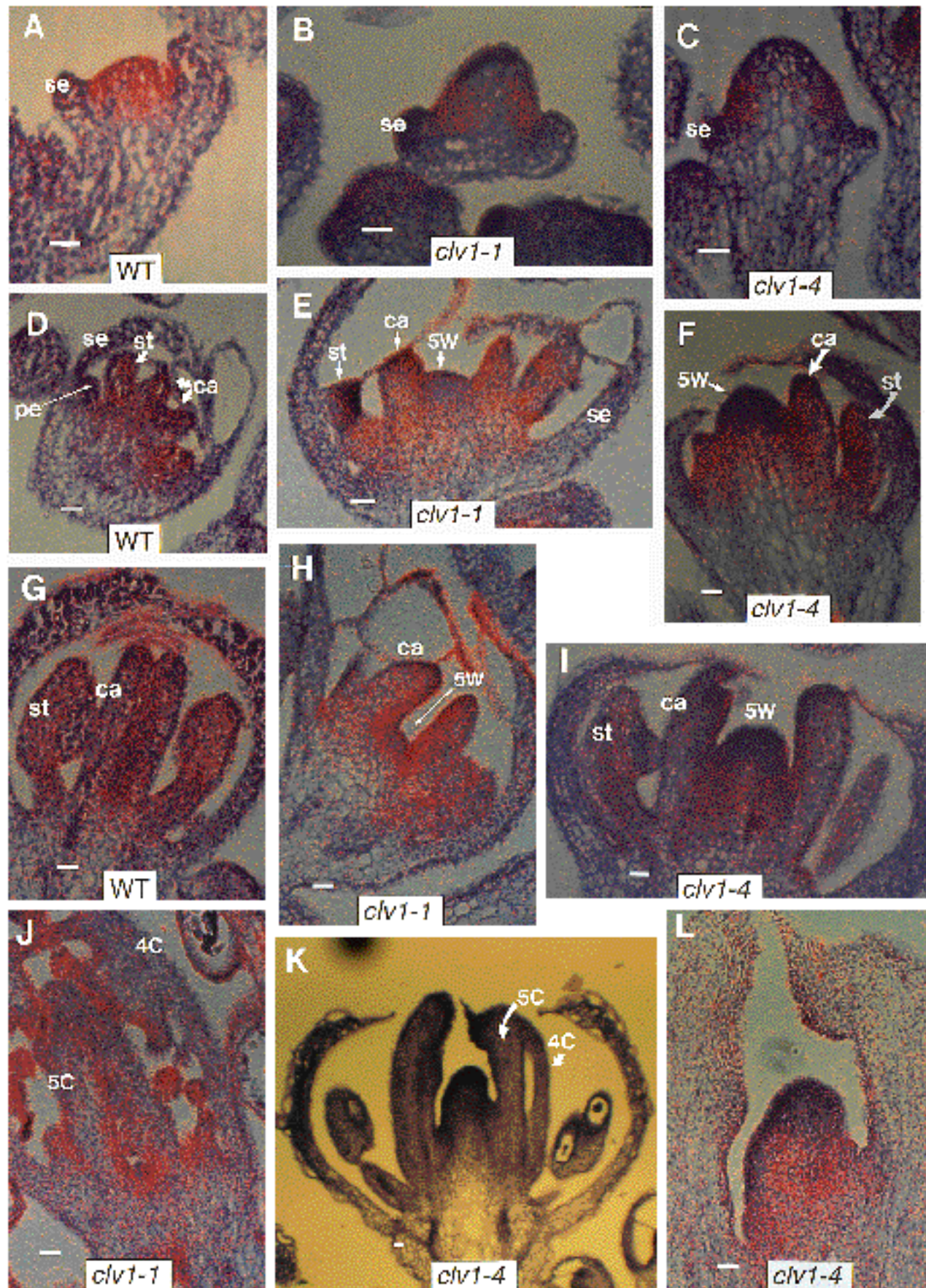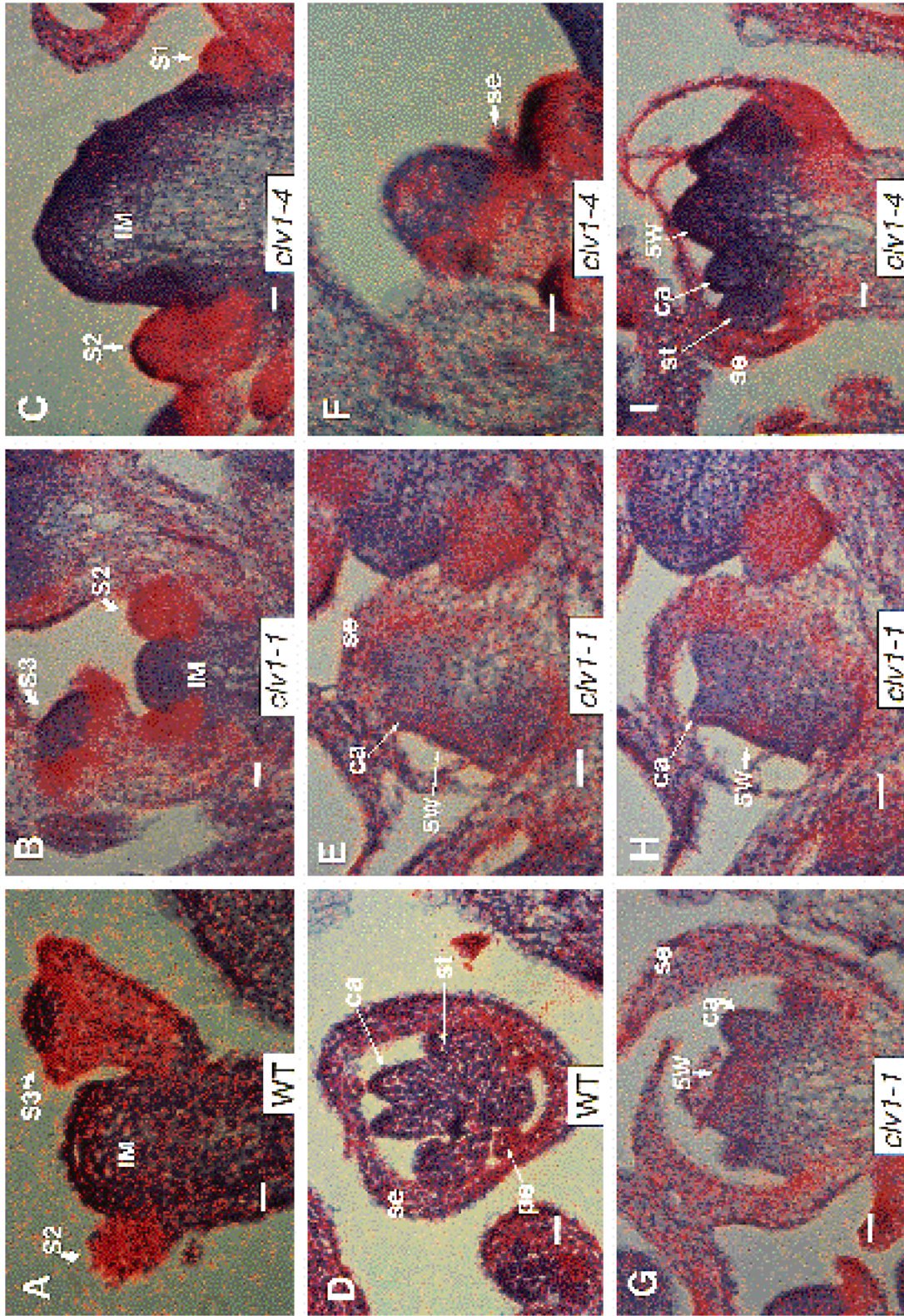
Fig. 10. For legend see p. 409

Fig. 11. *APETALA1* RNA expression in wild-type and *clavata1* plants. Landsberg (A,D), *clv1-1* (B,E,G,H) and *clv1-4* (C,F,I) inflorescences were fixed, sectioned and hybridized with labeled *AP1* probe as described (see Materials and methods). All panels are bright-field/dark-field double exposures. Dark-field exposures were taken with a red filter to visualize the signal. In wild-type, *clv1-1* and *clv1-4* plants, *AP1* RNA is expressed in the entire floral primordia of stage 1 (S1) and stage 2 (S2) flowers. In wild-type stage 3 flowers, *AP1* expression disappears from whorls 3 and 4 coincidental with the expression of *AG* there (A; see Fig. 10). As wild-type flowers develop, *AP1* expression remains restricted to sepals and petals (D). In *clv1-1* flowers, a low but consistent level of *AP1* expression is present in the fifth whorl of stage 5 and 6 flowers (E,G,H; note that E and H are sections of the same flower). This expression in the fifth whorl of *clv1-1* flowers is lost by stage 8. *clv1-4* flowers, on the other hand, clearly retain *AP1* expression in the center of stage 3 flowers (F), but this expression is lost by stage 5 (I). se, sepal; pe, petal; st, stamen; ca, carpel; 5W, fifth whorl; IM, inflorescence meristem; S1, S2 and S3, stage 1, 2 and 3 flowers, respectively. All plants carried the *erecta* mutation. Bars, 20 μm.
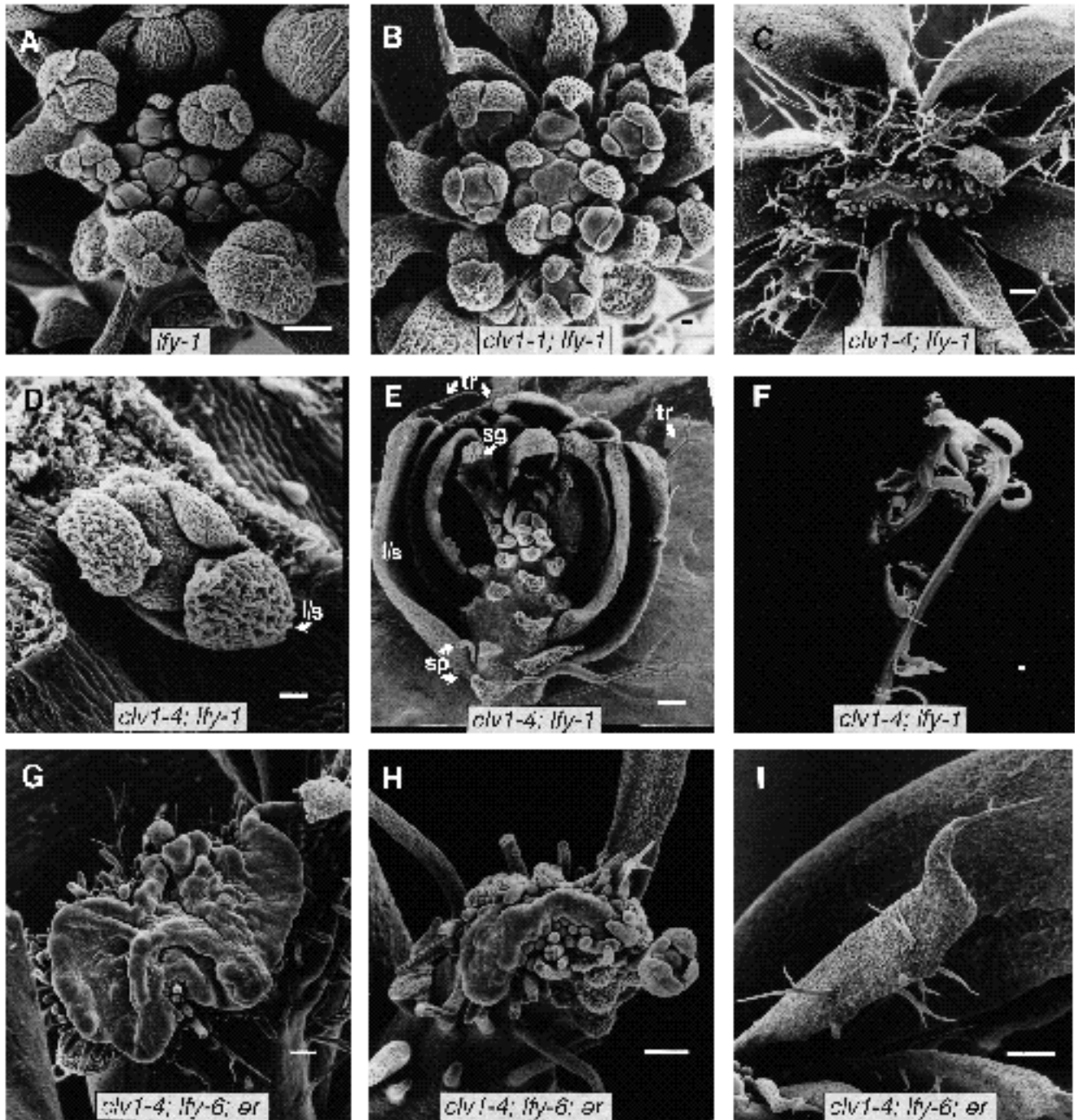
Fig. 12. *clavata1 leafy* double mutant plants. *lfy-1* (A), *clv1-1 lfy-1* (B), *clv1-4 lfy-1* (C-F) and *clv1-4 lfy-6 er* (G-I) plants were prepared as described and examined by SEM. Top views of inflorescence meristems of *lfy-1* (A) and *clv1-1 lfy-1* (B) reveal these plants generate flowers. A top view of a *clv1-4 lfy-1* inflorescence (C) meristem reveals only developing bracts and filamentous structures. Very young *clv1-4 lfy-1* flowers (D) exhibit complete spiral phyllotaxis of organ initiation. A *clv1-4 lfy-1* flower, from which several leaf-like sepals have been removed (E), displays many shoot characteristics (see text). A much older *clv1-4 lfy-1* flower (F) exhibiting continued floral proliferation is also shown. *clv1-4 lfy-6 er* apical meristems become massively proliferative (G), and *clv1-4 lfy-6 er* secondary inflorescence meristems become fasciated (H). In *clv1-4 lfy-1* and *clv1-4 lfy-6 er* plants, bracts with 'S' curve tips are occasionally observed (I). l/s, leaf-like sepal; sp, stipules; sg, stigmatic tissue; tr, stellate trichomes. Bar, 10 μm (D) and 100 μm for all other panels.

ovules on the margins of the carpelloid leaves (data not shown). Bracts with a 'S' curve at their tip were observed in *clv1-4 lfy-6 er* (Fig. 12I) and *clv1-4 lfy-1* plants.

clv1 ap1 lfy triple mutants

The flowers of *ap1-1 lfy-6* plants also display shoot characteristics: they develop spirally initiated organs, the flowers

are indeterminate, and the floral organs are either leaf-like or carpelloid (Weigel et al., 1992). Because *clv1-4 lfy-6* plants exhibit a flower-to-shoot transformation similar to that seen *ap1-1 lfy-6* plants, we sought to determine the role of *AP1* in the phenotype of *clv1-4 lfy-6* plants by examining *clv1-4 ap1-1 lfy-6* triple mutant plants. The apical meristems of *clv1-4 ap1-1 lfy-6* plants not only exhibited the overproliferation of the inflorescence meristem seen in *clv1-4 lfy-6* plants, but also developed tall meristem protrusions, often reaching 300 μm in height (Fig. 14B). When compared to *clv1-4 lfy-6* plants, *clv1-4 ap1-1 lfy-6* plants developed more bracts instead of filamentous organs, and more secondary inflorescences and 'flowers' in the axils of these bracts (Fig. 13), which suggests that *AP1* acts to suppress these structures in *clv1-4 lfy-6* plants. *clv1-4 ap1-1 lfy-6* flowers also exhibit a more complete transformation to shoot structures than *ap1-1 lfy-6* flowers (Fig. 14C,D). *clv1-4 ap1-1 lfy-6* secondary inflorescence meristems became fasciated (Fig. 14E), and *clv1-4 ap1-1 lfy-6* primary inflorescences occasionally developed bracts with 'S' curves at their tips (Fig. 14F).

DISCUSSION

We have investigated the effects of mutations in the *CLAVATA1* locus on *Arabidopsis* development. *clv1* mutant plants have enlarged apical and floral meristems, leading to a variety of phenotypes including fasciation, disrupted phyllotaxis, and extra floral organs and whorls. Double mutant combinations with the homeotic genes *AG*, *AP2*, *AP3* and *PI* reveal that *CLV1* controls the underlying floral meristem structure upon which the homeotic genes act. *CLV1* is the first *Arabidopsis* gene described in detail to affect floral meristem structure and floral organ number without affecting floral organ identity. *clv1* flowers also have altered patterns of *AG* and *AP1* expression. Mutant combinations with the meristem identity genes *AP1* and *LFY* reveal that *CLV1* also plays a role in the determination and maintenance of floral meristem identity. *CLV1* may also be necessary for the delayed transition to flowering under short day conditions.

CLV1 affects apical meristem structure

The *clv1* alleles differ in their effects on apical meristem structure. First, all *clv1* alleles enlarge the apical meristem during vegetative growth, although the strongest allele, *clv1-4*, causes noticeably greater enlargement than the other alleles. As the vegetative growth of the plant progresses, leaf primordia are generated much farther down from the tip of the apical meristem in *clv1* plants than in wild-type plants. As *clv1* plants make the transition from vegetative to floral growth, more phenotypic differences between alleles become clear. *clv1-5* inflorescence meristems (IMs) are simply enlarged compared to wild-type IMs. *clv1-1* IMs undergo fasciation, while *clv1-4* IMs can, especially at 16°C, develop into a massive meristem growth.

In the nineteenth century, a debate raged over the nature of fasciation. Linneaus had, in 1751, proposed that fasciation was the result of the fusion of buds resulting from crowding. His view was later supported by Hincks (1855)

and Masters (1869). Moquin-Tandon (1841) put forward a different model, namely that fasciation was caused by the transformation of a single growing point into a line. This model found many supporters including Nestler (1894) and Schoute (1936). It appears that in order to dismiss rare cases where stem fasciation was clearly the result of the fusion of buds (Franke, 1880), Nestler (1894) first proposed, and Schoute (1936) supported, the consideration of these cases as aberrations. In their definition, *true* or *typical fasciations* were those where fasciation occurred in accordance with their model (i.e. the transformation of a single growing point into a line), while any examples where the fusion of buds caused fasciation were not considered real fasciation, and were referred to as *connation*. While this distinction is artificial considering the lack of evidence on the origin of fasciation in the overwhelming majority of cases, it seems to have become accepted by at least one recent reviewer (Gorter, 1965). Our experiments give a detailed description of one type of inherited fasciation, which does not appear to occur by bud fusion. Morphologically, the *clv1* apical meristem is transformed from a point to a line (*clv1-1*) or a large mass (*clv1-4*). If we accept the classical view that the apical meristem is divided into a central zone (CZ) of slowly dividing cells that replenish a surrounding peripheral zone (PZ) where organ initiation occurs, then *clv1* apical meristems represent an enlargement of the CZ, because the region where organ initiation does not occur is much larger in *clv1* apical meristems than in wild type. However, it is curious that fasciation occurs in *clv1-1* not by simple enlargement of the apical tip, but by extensions of the apical meristem in the region where organs are being initiated (i.e. the putative PZ region). In addition, mutations in *LFY* and *AP1* enhance fasciation and meristem proliferation in *clv1* (Figs 12 and 14), despite the fact that these genes are not expressed in the apical meristem, but instead are expressed in nascent floral primordia.

CLV1 affects floral meristem identity

Double mutant combinations of *clv1* with *ap1* and *lfy* reveal that *CLV1* plays a previously unsuspected role in the determination and maintenance of floral meristem identity. In the case of *clv1 ap1* double mutants, nascent floral primordia are correctly specified, but cells in the center of these flowers can become specified as an inflorescence meristem. Thus, *CLV1* may contribute to the maintenance of floral meristem identity in a portion of the wild-type flower. This hypothesis is consistent with the observation that *clv1-4* flowers contain meristem-like tissue, though not an inflorescence meristem, at their centers. Because in *clv1-1* and *clv1-4* flowers *AP1* is expressed in cells that adopt an inflorescence meristem fate in *clv1 ap1-1* flowers, it is tempting to speculate that *AP1* expression maintains the floral meristem identity of the fifth whorl. Thus, when *AP1* activity is removed, these cells adopt an inflorescence meristem fate. This misexpression of *AP1* is likely due to the lack of *AG* expression in the center of *clv1* flowers, although it is interesting to note that the fifth whorl of stage 6 *clv1-4* flowers express neither *AG* nor *AP1*. The lack of *AG* and *AP1* expression in the center of *clv1-4* flowers may lead these cells to adopt a meristem fate. It is possible that the expression of *AP1* in the fifth whorl of stage 6 *clv1-1*
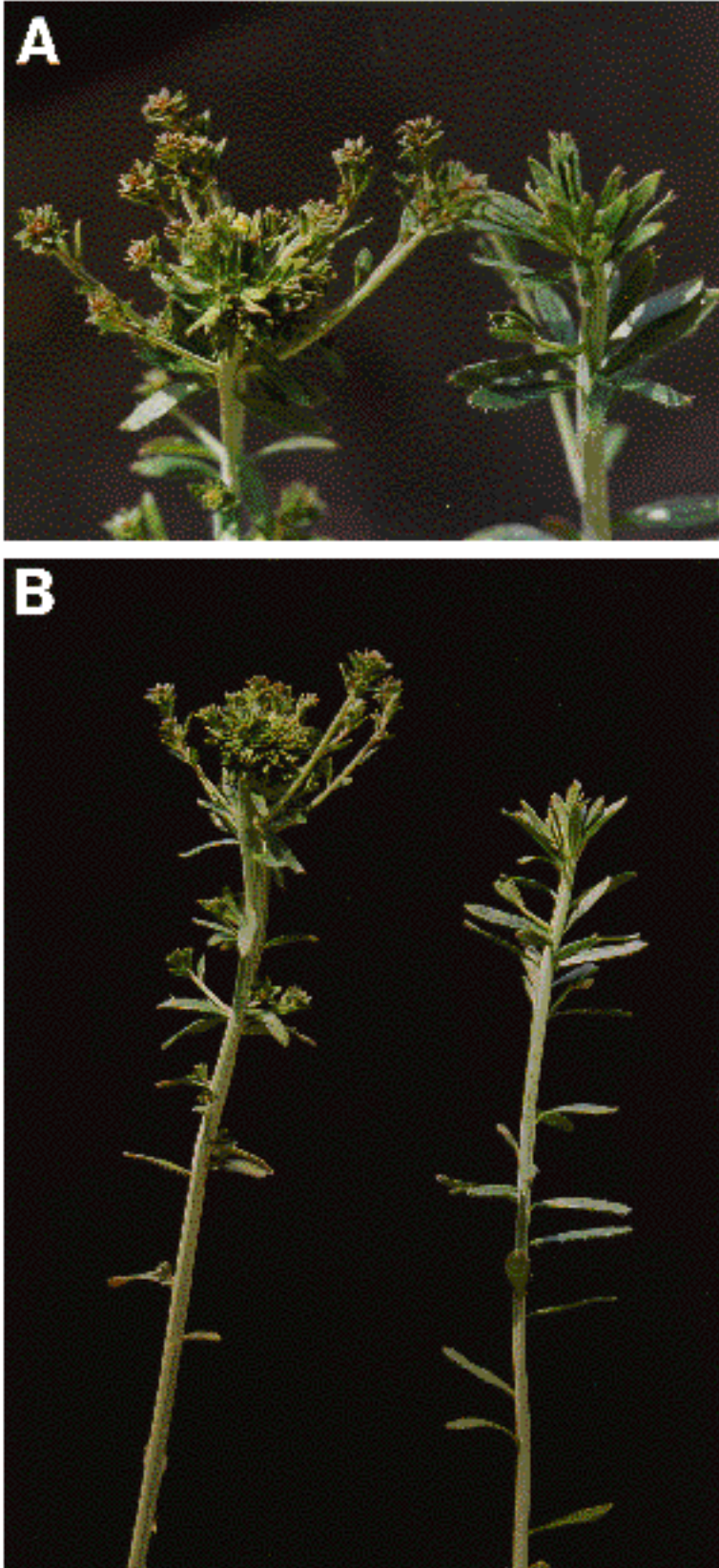
Fig. 13. Comparison of *clavata1 leafy* and *clavata1 apetala1 leafy* plants. *clv1-4 lfy-6* (right) and *clv1-4 ap1-1 lfy-6* (left) plants were grown as described and photographed. A shows a close-up of the top of the inflorescences, while B shows the entire inflorescence. *clv1-4 ap1-1 lfy-6* inflorescences generate more bracts, secondary inflorescences and transformed flowers than *clv1-4 lfy-6* inflorescences. Both plants carried the *erecta* mutation and were the same age.

flowers helps prevent further proliferation of these cells into the meristem-like tissue seen in *clv1-4* flowers (which do not exhibit this *AP1* expression). While *AP1*, as pointed out, is not expressed at the center of stage 6 *clv1-4* flowers, it is possible that its earlier misexpression in the center of stage 3 *clv1-4* flowers reduces the overall growth of these

cells, or reduces the number of cells adopting a meristem fate.

Another example of the role of *CLV1* in floral meristem identity is seen in the double mutant *clv1-4 lfy-1*. In this double mutant, very few floral primordia develop. Instead, most of the primordia generated by the inflorescence meristem are converted into filamentous organs and bracts. Those floral primordia that are generated develop into structures with very few floral characteristics and appear to be almost completely transformed into shoot meristems. The shoot characteristics of these flowers include spirally initiated organs, indeterminacy, and leaf-like organs with stipules and stellate trichomes. Thus the generation of flowers is almost blocked by mutations in these two genes. Hypotheses concerning the nature of *CLV1-LFY* interactions depend on the mechanism of *CLV1* action, and will be discussed below.

Further evidence of the role *CLV1* plays in floral meristem specification comes from observations of *clv1-4 ap1-1 lfy-6* triple mutant plants. Here we find that the flowers generated are more completely transformed into shoot structures than *ap1-1 lfy-6* flowers (Fig. 14). In addition, *clv1-4 ap1-1 lfy-6* plants develop more secondary inflorescences and transformed flowers than *clv1-4 lfy-6* plants (Fig. 13). This implies that the deficit in flower and secondary inflorescence development in *clv1-4 lfy-6* plants is not due to some defect in organ development caused by the *clv1-4* and *lfy-6* mutations, but rather appears to be the result of the suppression of these structures by *AP1* function.

CLV1 affects floral meristem structure

We have shown that *clv1* floral primordia are larger and develop more organs than wild-type flowers. *CLV1* is the first locus described to affect organ number and not organ identity. At the earliest stage of floral organ initiation (stage 3), the *clv1* floral meristem is already significantly taller than that of wild-type stage 3 flowers (Fig. 6). The enlargement of the portion of the floral meristem in *clv1* giving rise to whorls 3 and 4 in wild-type flowers correlates with and presumably causes the generation of additional organs in these whorls. Perhaps the anlage for whorls 3 and 4 are more affected than the anlage for whorls 1 and 2, because the *clv1* floral meristem is more affected in height than width. This might explain why whorls 3 and 4 exhibit a greater increase in organ number than whorls 1 and 2.

The mechanism responsible for floral meristem enlargement in *clv1* plants is unclear. Results of in situ hybridization experiments demonstrate that stage 3 *clv1* flowers lack *AG* expression at their center. Thus, one explanation for a taller floral meristem is that the absence of detectable *AG* expression in the center of the flower results in continued proliferation of these central cells. While this hypothesis would agree with observations that *AG* controls the determinacy of the floral meristem (Bowman et al., 1989), we consider it unlikely to be the only mechanism, for three reasons. First, the mutant floral meristem is already larger than the wild-type floral meristem at stage 3 when *AG* is first expressed. Second, *AG* is not expressed in the first or second whorls of *clv1* or wild-type flowers, yet these whorls exhibit extra organs in *clv1* flowers. Third, *ag* is not epistatic to *clv1*: *clv1-1* increases organ number in an *ag-2* background and

causes floral meristem fasciation, which is not seen in *ag-2* single mutants. These observations would suggest that *CLV1* controls floral meristem structure by acting independently as well as possibly acting through *AG*.

A second explanation for the enlarged floral meristem is that *clv1* floral meristems are initiated with more cells than wild-type floral meristems. This hypothesis would not explain, however, why any additional cells would continue to proliferate throughout floral development, and why those cells at the center of the floral primordia would not express *AG*, unless *AG* expression is triggered by some type of diffusion from the first or second whorl. A third explanation is that *clv1* fails to control cell proliferation in general. It is unclear, though, why only cells in the center of the floral meristem would continue to proliferate.

It is interesting that while the number of additional organs in each whorl is quite variable, and that in any given flower any of the four whorls can exhibit the greatest number of additional organs, very few mosaic or hybrid organs were observed (data not shown). This observation suggests that whorl boundaries are rigidly maintained despite the fact that each whorl has a variable number of extra organs, presumably resulting from having a variable number of extra cells. It is also of interest that these larger floral primordia give rise to more organs, and not bigger organs.

Another interesting phenotype of *clv1* flowers is the development of additional whorls interior to the fourth-whorl carpels. Despite the fact that wild-type flowers separate the floral primordia into only four whorls, the enlarged *clv1* floral primordia are divided into additional whorls, not just larger whorls. The flowers of weak and intermediate *clv1* alleles develop an additional whorl of carpels and then terminate, while *clv1-4* flowers often develop two additional whorls of carpels and continue to develop meristem-like tissue. Whether this meristem-like tissue is simply the result of floral indeterminacy, the result of a partial transformation of the center of *clv1-4* flowers into shoot identity, or something else entirely, is unclear.

CLV1 may be necessary for short day flowering delay

Normally, *Arabidopsis thaliana* grown under short day conditions experiences a significant delay in the transition to flowering (i.e. the vegetative growth phase is extended). While *clv1-1* plants exhibit this delay, *clv1-4* plants were unaffected by short day conditions. This difference in allelic response to short day conditions is likely due to the severity of the *clv1-4* allele, which exhibits clear differences from *clv1-1* for every trait examined. It is possible, though, that this is due to a separate, linked mutation that was not separated during the three back crosses. Mutations at other loci have been identified that result in early flowering, including *hy1*, *hy2*, *phyA* and *phyB*(*hy3*) (Goto et al., 1991; Zagotta et al., 1992; Reed et al., 1993). These loci are necessary for the generation of phytochrome A and B for the red light photoreceptors.

Mechanism of *CLV1* action

Mutations in *CLV1* disrupt floral meristem structure, inflorescence meristem structure, floral meristem identity and, apparently, timing of flowering under short day conditions.
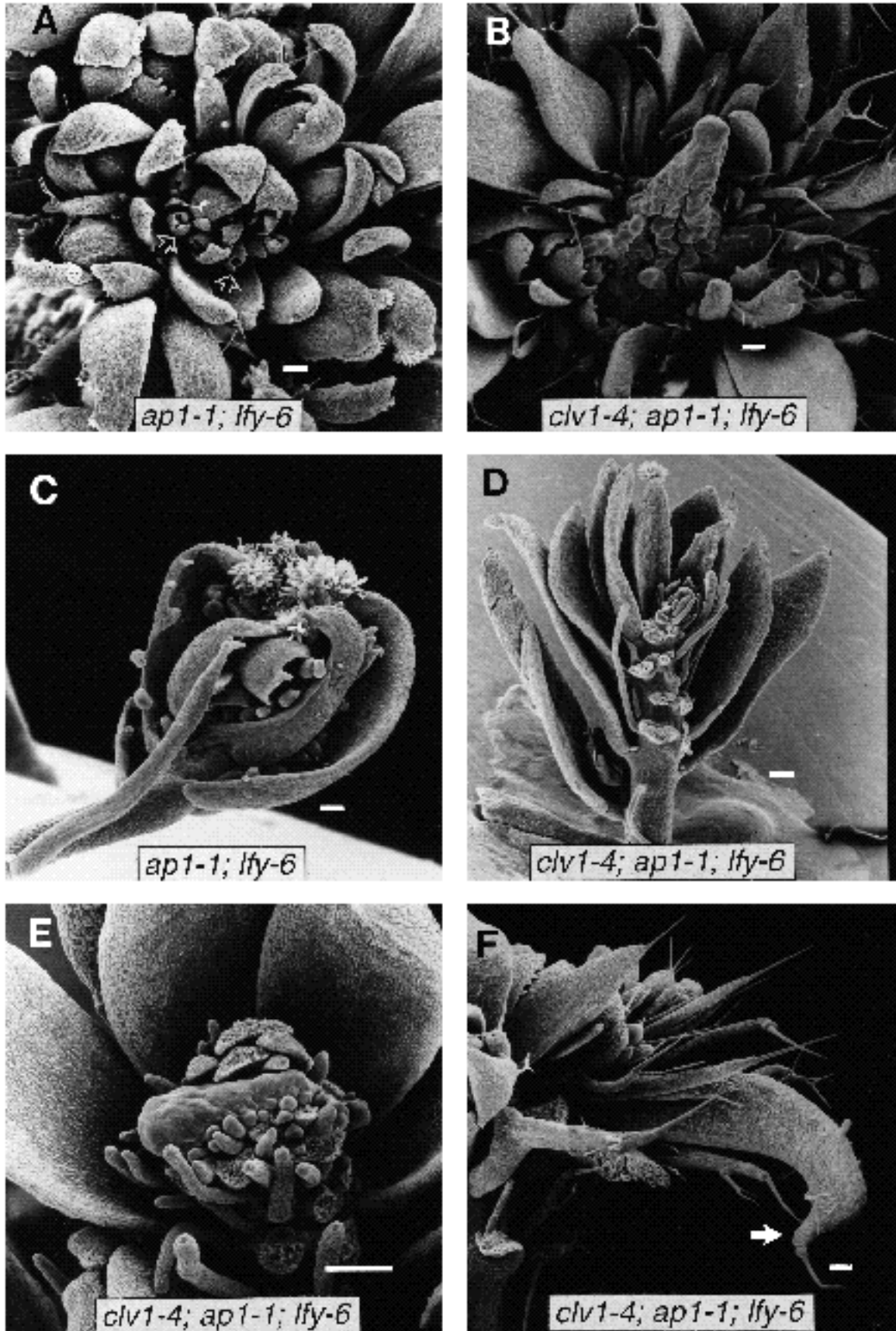
Fig. 14

Fig. 14. *clavata1 apetala1 leafy* triple mutants. *ap1-1 lfy-6* (A,C) and *clv1-4 ap1-1 lfy-6* (B,D,E,F) inflorescences were prepared as described and examined by SEM. (A,B) A comparison of inflorescence meristems of *ap1-1 lfy-6* and *clv1-4 ap1-1 lfy-6* plants shown at the same magnification. Note the developing flowers (open arrows in A), and the protruding meristem tissue in B. (C,D) A comparison of *ap1-1 lfy-6* and *clv1-4 ap1-1 lfy-6* flowers shown at the same magnification. *ap1-1 lfy-6* flowers are partially transformed into shoots, but in *clv1-4 ap1-1 lfy-6* flowers this transformation is more complete. *clv1-4 ap1-1 lfy-6* secondary inflorescence meristems become fasciated (E). *clv1-4 ap1-1 lfy-6* plants develop bracts with 'S' curve tips (F, closed arrow) similar to those found in *clv1-4 lfy-6* plants. All plants carried the *erecta* mutation. Bars, 100 μm.

One possible mechanism of *CLV1* action, which we consider unlikely, is that *CLV1* plays a separate role in each of these developmental pathways.

A second possibility is that *CLV1* plays a role in cell division patterns. If proliferation of cells in the center of apical and floral meristems were controlled by *CLV1* activity, *clv1* mutants could result in enlarged inflorescence meristems and flowers with proliferating, unspecified tissue in the center. In addition, the floral identity specification imparted on *lfy* floral primordia may require strict control of cell division patterns to compensate for loss of *LFY* activity, leading to an almost complete loss of floral specification in *clv1 lfy* double mutants. This model does not account for the possible *CLV1* function in short day flowering delay.

A third possibility is that *CLV1* plays a general role in signaling between plant cells. In this model, in developmental pathways in which this signaling is absolutely required, such as the establishment of inflorescence and floral meristem structure and the short day flowering delay, the role of *CLV1* would be revealed in the *clv1* single mutant. Other developmental pathways for which the *CLV1* signaling is not absolutely required, such as floral meristem identity, would reveal a *CLV1* role only when other possibly redundant components in the signaling pathway were mutated. This signaling could be long- or short-distance. For example, the role of *AP1* in *lfy* plants to establish partial floral meristem identity may require stage 1 flowers, expressing *AP1,* to send a *CLV1*-dependent signal to the adjacent floral anlage that would normally be expressing *LFY*, or may require cell-to-cell transmission within a single floral primordium to overcome the effect of the *lfy* mutation in particular cells. In these scenarios, *CLV1* would not be required for floral meristem specification in wild-type plants, but would be required for floral meristem specification in *lfy* plants. Neither of the last two possibilities exclude the other, and CLV1 may play a role both in specifying cell division patterns by affecting cellular signals, or vice versa.

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Note added in proof

Observations of additional carpel development interior to the four whorl gynoecium in *clv1* flowers has recently been noted by Shannon and Meeks-Wagner (*Plant Cell* **5**, 639-655).