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POLTERGEIST functions to regulate meristem development downstream of the *CLAVATA* loci

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SUMMARY

Mutations at the *CLAVATA* loci (*CLV1*, *CLV2* and *CLV3*) result in the accumulation of undifferentiated cells at the shoot and floral meristems. We have isolated three mutant alleles of a novel locus, *POLTERGEIST* (*POL*), as suppressors of *clv1*, *clv2* and *clv3* phenotypes. All *pol* mutants were nearly indistinguishable from wild-type plants; however, *pol* mutations provided recessive, partial suppression of meristem defects in strong *clv1* and *clv3* mutants, and nearly complete suppression of weak *clv1* mutants. *pol* mutations partially suppressed *clv2* floral and pedicel defects in a dominant fashion, and almost completely suppressed *clv2* phenotypes in a recessive manner. These observations, along with dominant interactions observed between the *pol* and *wuschel* (*wus*) mutations, indicate that *POL* functions as a critical regulator of meristem development downstream of the *CLV* loci and redundantly with *WUS*. Consistent with this, *pol* mutations do not suppress *clv3* phenotypes by altering CLV1 receptor activation.

Key words: Meristem, *POLTERGEIST*, *CLAVATA*, Phenotypic suppression, *Arabidopsis thaliana*

INTRODUCTION

The shoot meristem is the source of all above-ground adult organs in the plant. To function as a site of continuous organ formation, the shoot meristem must maintain a population of undifferentiated cells while directing descendant cells toward organ formation and eventual differentiation. A number of genes are specifically involved in maintaining the structure of the shoot and floral meristems in *Arabidopsis*. These include *SHOOT MERISTEMLESS* (*STM*), *WUSCHEL* (*WUS*) and the *CLAVATA* loci (*CLV1*, *CLV2* and *CLV3*) (Leyser and Furner, 1992; Barton and Poethig, 1993; Clark et al., 1993, 1995; Laux et al., 1996; Kayes and Clark, 1998). Analysis of genetic interactions between mutations at these loci indicate that several of these genes function in the same pathway and that all appear to regulate a similar process (for review see Clark, 1997).

clv1, *clv2* and *clv3* mutants accumulate undifferentiated cells at both the shoot and floral meristems (Clark et al., 1993, 1995). In the *clv* flowers, the larger meristem leads to the initiation of additional organs in each whorl, and extra whorls of organs interior to the normally terminal whorl 4 carpels. In plants homozygous for the strongest *clv1* and *clv3* alleles, the floral meristem is indeterminate. *clv1* and *clv3* mutants exhibit identical phenotypes, are mutually epistatic, and exhibit dominant interactions, indicating that the *CLV1* and *CLV3* genes function in the same pathway (Clark et al., 1995). *clv2* mutants exhibit only weak phenotypes at the shoot and floral meristem compared to *clv1* and *clv3* mutants (Kayes and Clark, 1998). *clv2* mutants also affect the development of several organ types, including pedicels, stamens and gynoecia. Genetic analysis indicates that *CLV2* functions in the same pathway as *CLV1* and *CLV3* to regulate meristem development, but in an independent pathway to regulate organ development (Kayes and Clark, 1998).

wus and *stm* mutants fail to maintain a population of undifferentiated cells at the shoot and floral meristems (Barton and Poethig, 1993; Laux et al., 1996). This results in prematurely terminated shoot and floral meristems, as well as reduced numbers of floral organs, especially the central stamens and carpels. The strongest *stm* mutants fail to initiate any shoot meristems, while plants homozygous for the weaker *stm* alleles initiate shoot meristems that are overtaken by organ primordia (Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996). *wus* mutants, in contrast, form meristem-like structures that differentiate across the apex (Laux et al., 1996). *wus* and *stm* mutants also display differences in genetic interactions with *clv* mutants. *wus* is epistatic to *clv1* and *clv2* mutations (Laux et al., 1996), while *clv stm* double mutant plants exhibit an additive phenotype (Clark et al., 1996). The dominant interactions between *clv* and *stm* mutations suggest that they act competitively on a common downstream target. However, the epistasis of the *wus* mutation indicates that either *WUS* functions downstream of the *CLV* loci, which act to negatively regulate *WUS*, or *WUS* functions to establish the meristem upon which the *CLV* loci act. Both *STM* and *WUS* code for homeodomain-containing proteins, and are expressed within a central region of the shoot and floral meristems (Long et al., 1996; Mayer et al., 1998).

To expand our understanding of the hierarchy of genes regulating meristem development, we undertook a screen for second-site mutations that enhanced or suppressed the phenotypes of intermediate *clv1* and *clv3* mutants. We identified three mutant alleles of a novel locus we term *POLTERGEIST* (*POL*) (Pogany et al., 1998). Here we describe in detail a morphological and genetic analysis of *pol* mutant plants. While *pol* single mutant plants were nearly identical to wild type, *pol* provided recessive, partial suppression of the phenotypes of strong *clv1* and *clv3* mutants, and nearly complete suppression of weak *clv1* mutants. *pol* mutations partially suppressed *clv2* phenotypes in a dominant manner, and almost completely suppressed *clv2* phenotypes in a recessive manner. *wus* became semi-dominant in a *pol* background, and *pol* enhanced *wus* phenotypes, suggesting that *POL* functions redundantly with *WUS*. Finally, *pol* mutations did not suppress *clv3* phenotypes by altering CLV1 receptor-kinase activation.

MATERIALS AND METHODS

Plant growth and genetic techniques

The isolation of the *pol-1*, *pol-3* and *pol-4* mutations was described previously (Pogany et al., 1998).

Seeds were sown on a 1:1:1 mix of top soil:perlite:vermiculite and imbibed for 7 days at 4°C. Plants were grown at 22°C under approximately 800 foot-candles of constant cool white fluorescent light. Plants were given fertilizer once a week.

The isolation of *pol wus*, *pol stm*, and *pol ag* double mutant plants were carried out as follows. *pol clv3* double mutant plants were crossed to *stm/STM* and *wus/WUS* single mutant plants, and *pol clv1* double mutant plants were crossed to *ag/AG* and *wus/WUS* single mutant plants. Progeny were collected from individual F_1 plants, and those F_2 populations segregating out all three mutant phenotypes were used for further analysis (Fig. 1). Progeny exhibiting wild-type phenotypes were collected from individual F_2 plants, and the segregation of the mutations in the F_3 generation was assessed. F_3 families segregating only wild-type, suppressed *clv*, and the other mutant-like phenotypes (*ag*, *wus*, or *stm*) were followed for subsequent analysis. The parent F_2s for these families were homozygous for *pol*, and heterozygous for *clv* and the other mutation. Progeny were collected from wild-type individuals in these F_3 families, and the F_4 generation was assessed. F_4 families that segregated out only wild type and the mutation in question were from F_3 individuals that were homozygous for *pol*, homozygous wild-type for *CLV*, and heterozygous for the mutation in question. The number of families in each phenotypic class is indicated in Table 1. For *ag*, additional steps were taken to ensure the identity of the *pol ag* double mutant, because plants heterozygous for *ag* exhibit phenotypes similar to *pol clv* double mutant plants (i.e., a low frequency of flowers with extra carpels). To further verify that the putative *pol ag* double mutant plants were homozygous for *pol*, two addition assays were performed. First, the 90J19T7 and additional markers both north and south of *POL* on chromosome 2 were tested. The *pol ag* double mutants were all homozygous for the *pol-1* (Columbia) polymorphism at these markers. Second, the pedicels of *pol ag* double mutant plants were compared to pedicels of *ag* single mutants, and the *pol ag* double mutants had significantly shorter pedicels (6.48±0.61 mm/*ag-3* pedicel vs. 4.93±0.12 mm/*pol-1 ag-3* pedicel).

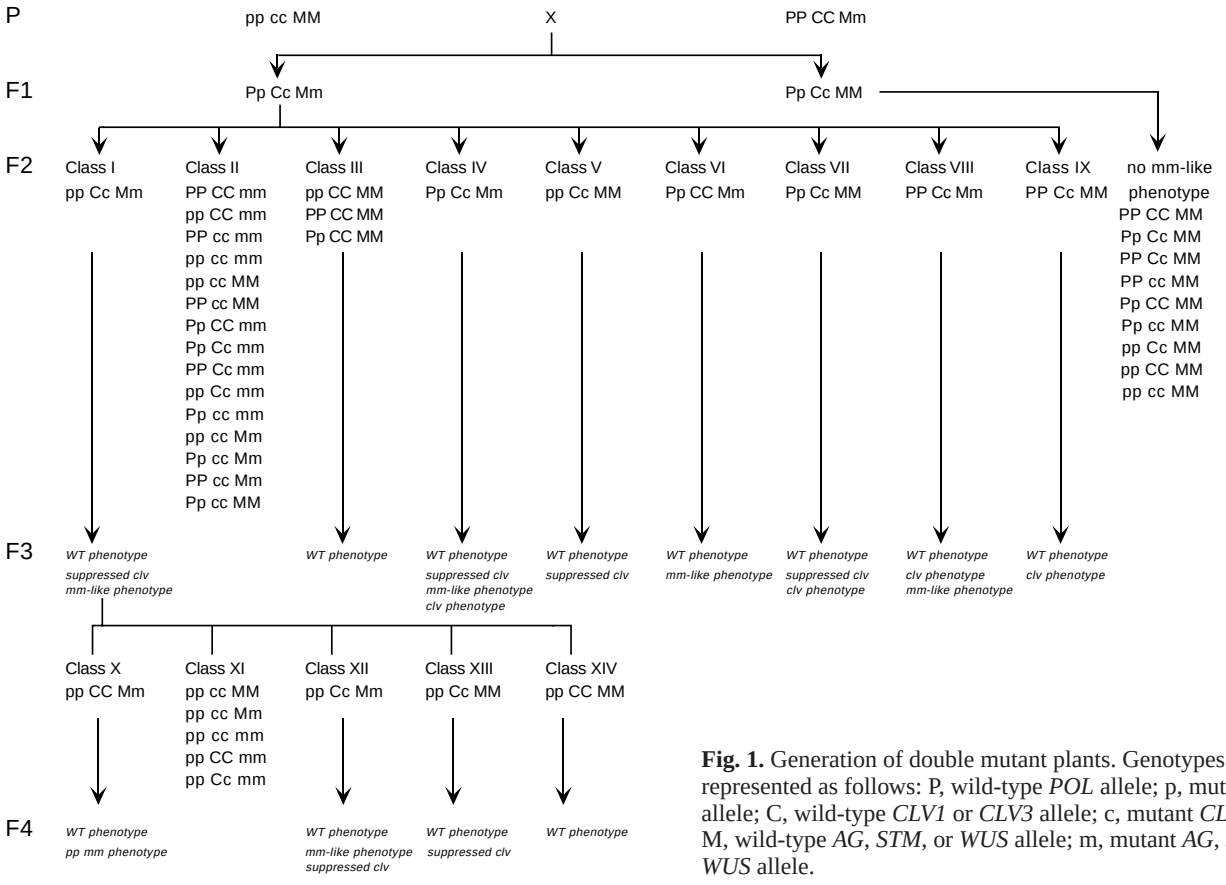

Fig. 1. Generation of double mutant plants. Genotypes are represented as follows: P, wild-type *POL* allele; p, mutant *POL* allele; C, wild-type *CLV1* or *CLV3* allele; c, mutant *CLV* allele; M, wild-type *AG*, *STM*, or *WUS* allele; m, mutant *AG*, *STM*, or *WUS* allele.

Table 1. Generation of *pol-1* double mutants

Class of plants*	Number of plants			
	pol-1 clv1-4 × wus-1/WUS	*pol-1 clv3-1 × wus-1/WUS*	*pol-1 clv1-4 × stm-1/STM*	*pol-1 clv3-1 × stm-2/STM*
I	3	7	10	2
III, IV, V, VI, VII, VIII, IX	27	21	34	30
X	9	9	14	14
XII, XIII, XIV	18	39	51	32

*See Fig. 1 for an explanation of the different classes.

Mapping

The *clv3-1* line that was originally mutagenized to generate the *pol-1* allele was a Columbia/Landsberg *erecta* mixture. Preliminary mapping in crosses with known genetic markers indicated that *pol* mapped to the bottom arm of chromosome 2 (data not shown). In the original *clv3-1* line, the entire bottom arm of chromosome 2 was homozygous for Columbia polymorphisms. Thus, we used *pol-1* that had been backcrossed two times to *clv1-1* (Landsberg) for mapping. DNA was collected from each F_2 plant from the final cross, and the genotype of the plant was assessed by analyzing F_3 progeny. 477 progeny were assayed for CAPS markers on the bottom arm of chromosome 2. Tightest linkage was observed with marker 90J19T7, which showed only 5 recombination events over 954 chromosomes. Thus, *pol* is tightly linked to this marker.

Tissue and image processing

Scanning electron microscopy (SEM) was carried out as described by Bowman et al. (1989), except the Hitachi S3200N SEM allowed images to be collected digitally. Slides were scanned and digitized using a Polaroid SprintScan35. Brightness, contrast, and color balance were adjusted using Adobe Photoshop and figures were printed using a Kodak 8600 Digital Printer. *pol* and wild-type plants were fixed in formaldehyde, acetic acid and ethanol mixture (Clark et al., 1997), embedded in paraffin, and sectioned at 4 μm thickness.

Floral stages

In order to clearly categorize the stages of floral primordia, physical characteristics were defined for stages 1, 2, 3 and 4 flowers based on those of Smyth et al. (1990). Stage 1, primordia have formed a furrow between the shoot meristem and primordia; stage 2, the floral meristem physically contacts the inflorescence meristem; stage 3, floral meristems have formed sepal primordia; stage 4, the sepals physically contact the floral meristem prior to the formation of stamen primordia (Fig. 2C).

Analysis of shoot meristem size

Scanning electron micrographs were taken from directly above the shoot meristem. The center of the furrow of the youngest stage 1 floral primordium was determined with a compass. A line connecting the furrow ends was drawn. The distance from the center of the furrow line perpendicularly across the shoot meristem was defined as the size of the shoot meristem (Fig. 2C).

RESULTS

Mutant isolation

Ethylmethane sulfonate mutagenesis was performed on the intermediate *clv3-1* and *clv1-1* mutant plants to identify enhancers and suppressors (Pogany et al., 1998). Three independent families segregated a recessive suppressor of the *clv* mutation (Fig. 2). When the suppressed *clv1-1* plants were backcrossed to wild type, an approximate 12:3:1 ratio of

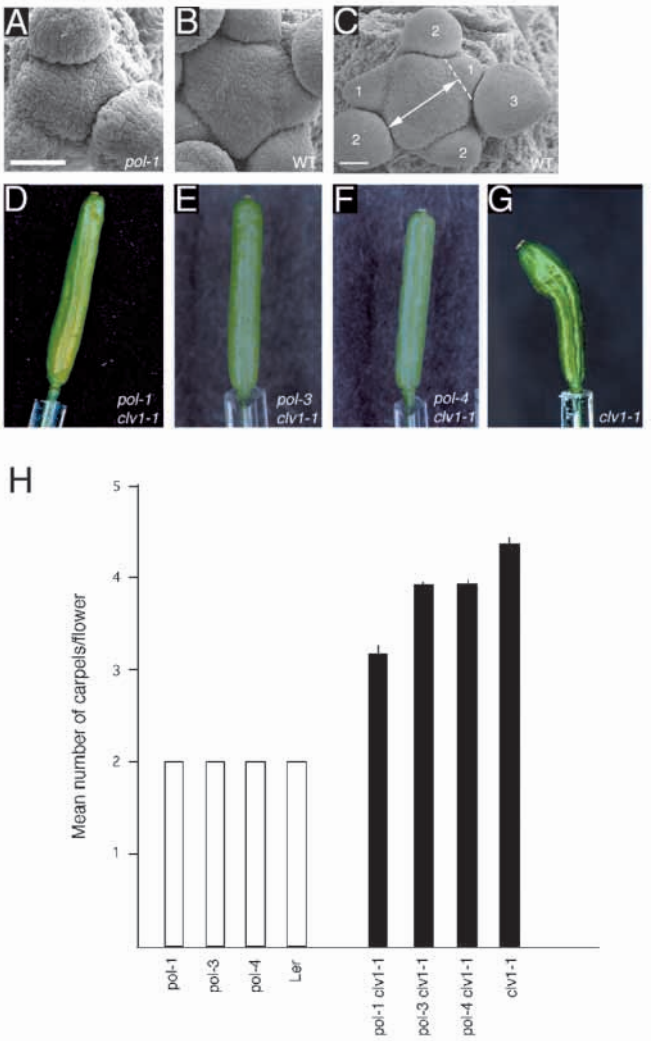

Fig. 2. *pol* mutant plants are similar to wild type, and *pol* alleles are able to partially suppress the *clv1-1* mutant phenotypes. Scanning electron micrographs of inflorescence shoot meristems of *pol-1* (A) and wild-type (WT) Landsberg (B,C), and photos of gynoecia of *pol-1 clv1-1* (D), *pol-3 clv1-1* (E), *pol-4 clv1-1* (F), and *clv1-1* (G). A,B are shown at the same magnification, as are D-G. (C) Dotted line represents line drawn through the furrow ends for measurement of shoot meristem (see Materials and Methods). Double-headed arrow indicates the length of the shoot meristem size. Scale bar, 25 μm. (H) Values represent the mean number of carpels for *pol-1, pol-3, pol-4,* wild-type Landsberg (Ler) , *pol-1 clv1-1*, *pol-3 clv1-1*, *pol-4 clv1-1*, and *clv1-1*. 100 flowers were counted for each mean calculated. Only the first ten flowers and pedicels of any given plant were analyzed. Standard errors of the mean are indicated by vertical black lines.

(wild-type phenotype):(clv1 phenotype):(suppressed clv1 phenotype) plants were observed in the F_2 generation (data not shown). Thus, we suspected that plants with the suppressing mutation(s) alone were indistinguishable from wild type. Seeds were collected from F_2 individuals and progeny were scored for clv1 phenotypes. Approximately two-thirds of the plants phenotypically clv1 gave rise to progeny in which a 3:1 ratio of (clv1 phenotype):(suppressed clv1 phenotype) was observed, indicating that the suppression was caused by a single, recessive mutation. Among the phenotypically wild-type plants, a portion gave rise to progeny with a 3:1 ratio of (wild-type phenotype):(suppressed clv1 phenotype). We concluded that these plants were homozygous for the suppressor mutation. By collecting progeny from these F_3 individuals, we were able to isolate lines that were both homozygous for the suppressing mutation and wild type for *CLV1*. These plants were indistinguishable from wild type (or nearly so – see below). Because the mutation could not be identified in an otherwise wild-type plant, we termed the original suppressor allele *poltergeist* (*pol*). Prior to further phenotypic and genetic analysis, the *pol-1 clv* double mutant plant was backcrossed to wild type three times.

Allelism tests were performed by crossing a *pol-1* single mutant plant with other suppressed *clv1-1* plants. For the mutations that were *pol* alleles, these crosses resulted exclusively in wild-type and suppressed clv1 phenotypes in both the F_2 and F_3 generations.

For *pol-1*, which was isolated as a suppressor of *clv3-1*, we observed linkage between the *pol* and *clv3-1* mutations (data not shown). Fine mapping on a large population revealed only 5 recombination events between *pol* and CAPS marker 90J19T7 on the bottom arm of chromosome 2 over 954 chromosomes (see Materials and Methods), indicating *pol* is tightly linked to this marker.

The three different *pol* alleles were analyzed for the relative severity of the mutant phenotype and the effect of *pol* on meristem development. Plants homozygous for *pol* alleles developed flowers with an identical number of floral organs as wild type (Table 2). To compare the relative strength of the *pol* alleles, the reduction in carpel number in the *pol clv1-1* double mutant plants was determined (Fig. 2H). The *pol-1* allele provided the greatest suppression of *clv1-1*, and was therefore used for all subsequent genetic and phenotypic analyses.

While visible inspection of *pol* single mutant plants revealed no shoot meristem abnormalities, scanning electron microscopy (SEM) analysis of *pol* shoot meristems revealed a possible slight reduction in meristem size (Fig. 2A,B). To determine whether the *pol* meristem was reduced compared to wild type Landsberg, the meristem sizes of 18 wild-type and *pol-1* plants were determined by SEM (Fig. 2C; Table 3; see Materials and Methods). While the mean *pol-1* meristem size was smaller than that of wild type, the average difference was only 5 μm. We also compared *pol* and wild-type meristem size by longitudinal serial sections of their apices (Fig. 3). Fewer sections were required for *pol* meristems and they were narrower than wild type. To confirm this reduction by a different measurement, we determined the mean number of stage 1, 2, 3 and 4 flowers present on *pol-1* inflorescence shoot meristems. We hypothesized that a smaller meristem would initiate organs at a slower rate. We observed a slight reduction in the number of stage 1-4 flowers in *pol* mutants (7.7 / *pol* meristem vs. 9.0 / wild-type meristem; Table 3), which may indicate a reduced shoot meristem size, or a faster rate of *pol* flower development.

Table 2. *pol* suppresses the floral organ number defects of *clv* mutants

Genotype	Mean number of organs per flower			
	Sepals	Petals	Stamens	Carpels
Wild type (L*er*)	4.00±0	4.00±0	5.55±0.06	2.00±0
pol-1	4.00±0	4.00±0	5.54±0.06	2.00±0
clv1-6	4.07±0.03	4.10±0.03	6.28±0.06	3.91±0.04
pol-1 clv1-6	4.00±0	3.98±0.02	6.01±0.01	2.04±0.02
clv1-1	4.28±0.05	4.29±0.05	6.82±0.07	4.36±0.06
pol-1 clv1-1	4.01±0.01	4.01±0.01	6.03±0.02	3.17±0.08
clv1-4	4.92±0.07	4.56±0.07	7.19±0.09	5.12±0.08
pol-1 clv1-4	4.04±0.02	4.03±0.02	6.10±0.04	3.90±0.07
clv2-2	4.02±0.01	4.02±0.01	5.92±0.04	3.91±0.03
pol-1 clv2-2	4.00±0	3.99±0.01	5.41±0.07	2.02±0.01
clv2-3	4.22±0.05	4.15±0.04	6.62±0.06	3.74±0.05
pol-1 clv2-3	4.00±0	4.00±0	5.55±0.06	2.04±0.02
clv2-4	4.23±0.05	4.22±0.05	6.43±0.07	3.25±0.11
pol-1 clv2-4	4.05±0.02	4.05±0.02	5.59±0.06	2.54±0.07
clv3-1	4.56±0.06	4.52±0.06	7.73±0.09	4.86±0.10
pol-1 clv3-1	4.00±0	4.00±0	6.05±0.03	3.46±0.08
clv3-2	4.96±0.05	4.95±0.19	7.66±0.09	5.80±0.09
pol-1 clv3-2	4.02±0.01	4.03±0.02	6.07±0.04	3.74±0.06

Values represent the mean number ± standard error of the mean of indicated floral organs. 100 flowers were counted for each mean calculated. Only the first ten flowers of any given plant were analyzed.

Whorl 3 organs that developed as filamentous organs were included in the counts of stamens.

Genetic interactions with *clv* mutations

To determine the position of *POL* within the hierarchy of genes regulating meristem development, double mutant plants were first generated between *pol-1* and *clv1*, *clv2* and *clv3* mutations (Fig. 4; Table 2). Plants homozygous for the strong *clv1-4* and *clv3-2* alleles give rise to dramatically enlarged shoot and floral meristems (Clark et al., 1993) (Fig. 4G,O). The *pol-1 clv1-4* and *pol-1 clv3-2* double mutant plants exhibited a clv

Table 3. Floral initiation and shoot meristem size are reduced in *pol-1* mutants

	Mean number of floral primordia per meristem*·‡	
	Landsberg	*pol-1*
Floral stage§		
1	1.78±0.25	1.78±0.10
2	2.89±0.14	2.72±0.16
3	1.94±0.20	1.44±0.12
4	2.39±0.12	1.78±0.13
total	9.00±0.33	7.72±0.24
Shoot meristem size (μm)¶	75.75±2.74	70.14±2.05

*Samples were collected 28 days after germination. 18 samples were counted for each mean calculated.

‡Values represent the mean number of floral primordia ± standard error of the mean.

§See Material and Methods for definition of floral stages.

¶Values represent the mean shoot meristem size (μm) ± standard error of the mean.

Table 4. *pol-1 clv* double mutant phenotypes

	clv1-6 *clv1-7*	*clv2-1* *clv2-2* *clv2-3* *clv2-4*	*clv1-1*	*clv3-1* *clv1-8*	*clv1-4* *clv3-2*
Strength of clv phenotype*	Weak	Weak intermediate	Intermediate	Strong intermediate	Strong
Strength of pol clv phenotype*	Wild type‡	Wild type‡	Weak	Weak	Weak intermediate

*The phenotypes of the *pol-1 clv* and *clv* mutant plants are indicated. Phenotype classification is based on the extent of shoot meristem proliferation, the number of floral organs, and the extent of floral indeterminacy. See Table 2.
‡Mean number of floral organs was nearly identical to wild type.

phenotype, but it was strongly suppressed as measured by shoot meristem size, the number of floral organs, which is indicative of floral meristem size (Clark et al., 1993), and the extent of indeterminacy at the floral meristem (Fig. 4G,H,O,P; Table 2; data not shown). This recessive suppression resulted in plants similar to plants homozygous for weak *clv* alleles (Fig. 4; Tables 2, 4).

When double mutant plants were generated between *pol-1* and weaker *clv1* alleles (*clv1-6* and *clv1-7*), a nearly complete suppression was observed (Tables 2, 4). The mean number of floral organs in these double mutants varied only slightly from wild type (Table 2; data not shown). Similarly, the shoot meristems of the *pol-1 clv1-6* and *pol-1 clv1-7* double mutants were reduced in size to that seen in wild-type plants (Fig. 4A,B; Table 4). To determine if *pol* could provide semi-dominant suppression of *clv1-1* mutant phenotypes, the progeny of *pol-1*/*POL clv1-1*/*clv1-1* plants were scored for the number of carpels in each of the first ten flowers (Fig. 5B). Each plant was then tested for the *pol* genotype (*pol*/*pol*, *pol*/*POL*, or *POL/POL*) by examining progeny. Mean carpel number for *pol*/*POL* heterozygotes was less than *POL*/*POL* homozygotes, indicating that *pol* suppression of *clv1-1* was semi-dominant.

clv2 mutant plants are somewhat different than *clv1* or *clv3* mutant plants in that: (1) all *clv2* mutant plants exhibit weak phenotypes despite being putative nulls (Jeong et al, 1999); (2) clv2 phenotypes, but not clv1 or clv3 phenotypes, are altered under short-day photoperiod conditions; and (3) *CLV2* plays a role independent from *CLV1* and *CLV3* to regulate the development of several organ types (Kayes and Clark, 1998). One of the organ types regulated by *CLV2* is the floral pedicel. In *clv2* mutant plants, pedicels are on average approximately 50% longer than wild type (Kayes and Clark, 1998). *clv2* mutants under short-day photoperiod develop wild-type flowers with elongated pedicels, indicating that the regulation of pedicel length by *CLV2* is independent of its regulation of the floral meristem.

We sought to determine whether *pol* was capable of suppressing *clv2* meristem and/or organ defects. Double mutant plants were generated between *pol-1* and *clv2-1*, *clv2-2*, *clv2-3* and *clv2-4*. Similar to the double mutant plants of *pol* with weak *clv1* alleles, the *pol clv2* double mutant plants were nearly identical to wild type in terms of the size of the shoot meristem and the number of floral organs (Fig.

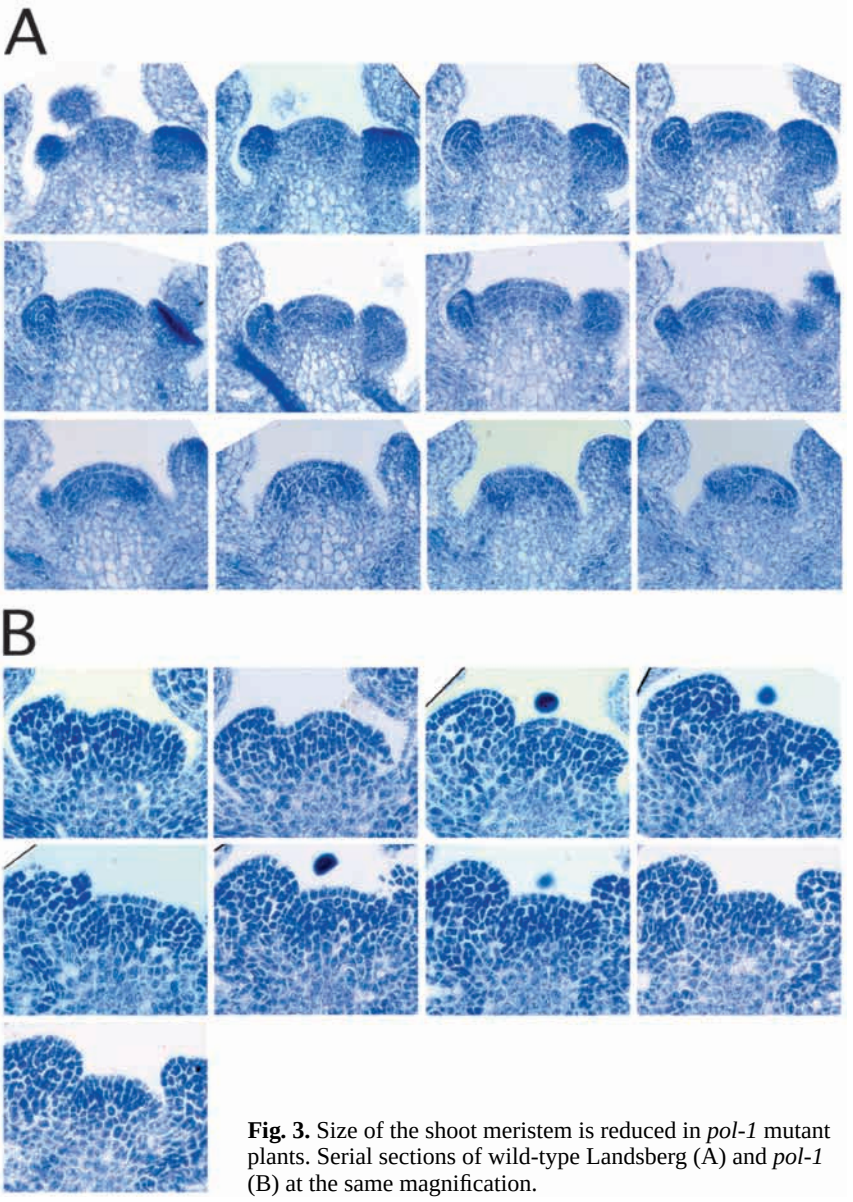

Fig. 3. Size of the shoot meristem is reduced in *pol-1* mutant plants. Serial sections of wild-type Landsberg (A) and *pol-1* (B) at the same magnification.

4E,F,I,J,M,N; Tables 2, 4). The mean number of floral organs varied only slightly from those of wild type (Table 2). Interestingly, *pol* also suppressed the elongation of the pedicel caused by *clv2* mutations (Fig. 5A). This prompted us to examine the length of pedicels in *pol* single mutant plants compared to wild type, which revealed that *pol* mutant plants developed significantly shorter pedicels than wild type (Fig. 5A).

While generating the *pol clv2* double mutant plants, we observed that a significant portion of the progeny of *pol*/POL *clv2*/*clv2* plants exhibited partial suppression. To determine if this was the result of semi-dominant suppression of *clv2* by *pol*, each individual plant was scored for floral organ number and pedicel length, and subsequently scored for *pol* genotype by testing the progeny. *pol* heterozygotes were significantly reduced in both floral organ number and pedicel length compared to plants wild-type for *POL* (Fig. 5). Thus, *pol* was semi-dominant in its suppression of *clv2*, and suppressed both meristem and organ defects.

pol suppression of indeterminacy is specific to *clv* mutants

As mentioned above, *pol* suppressed the indeterminate floral meristems that develop in strong *clv1* and *clv3* mutant plants. To determine if this suppression was specific for the *CLV* loci, we tested the ability of *pol* to suppress the indeterminate *agamous* (*ag*) floral meristem. *ag* mutations result in the replacement of stamens by petals, and a new flower in the place of carpels (Bowman et al., 1989). Thus, the *ag* flower initiates an indeterminate series of (sepals, petals, petals)$_n$. We generated *pol ag* double mutant plants, by crossing *pol-1 clv1-4* to *ag-3/AG*. By testing large numbers of F_2 progeny, we identified a plant *pol-1*/*pol-1 clv1-4*/*CLV1 ag-3*/*AG* (Fig. 1; see Materials and Methods). By collecting seeds from a number of progeny of this plant, we isolated a plant *pol-1*/*pol-1 CLV1*/*CLV1 ag-3*/*AG*, and compared the progeny of this plant to the progeny of an *ag-3*/*AG* plant. We observed no difference in the development of the *ag-3* and *pol-1 ag-3* flowers (Fig. 6), indicating that *pol* had no effect on the indeterminacy found in *ag* flowers.

wus-pol dominant interactions

To better place *POL* within the hierarchy of genes regulating meristem development, we also investigated interactions between *pol* and *wus* mutations. *wus* mutants fail to maintain organogenesis at both the shoot and flower meristems (Laux et al., 1996). This results in the repeated initiation of adventitious meristems that quickly terminate, and flowers that largely lack central organs (stamens and carpels). *pol-1 wus-1* double mutant plants were isolated in a manner similar to the isolation of the *pol ag* double mutant plants (Fig. 1; Table 1; see Materials and Methods). In comparison to *wus* single mutant plants, the *pol wus* plants exhibited less overall growth, reduced plant stature, abnormal leaf development, and a reduced number of flowers (Fig. 7A). A comparison of the number of floral organs initiated by *wus* and *pol wus* flowers revealed that *pol* enhanced the reduction in floral organ number observed in *wus* mutants (Table 5). In addition, we observed among the progeny of *pol-1 wus-1*/*pol-1 WUS* plants a significant portion of plants that exhibited weak *wus* phenotypes. These phenotypes consisted primarily of terminated vegetative shoot meristems, but rarely the termination of inflorescence shoot meristems as well (Fig. 7B-E). To determine if the terminated meristem phenotype was the

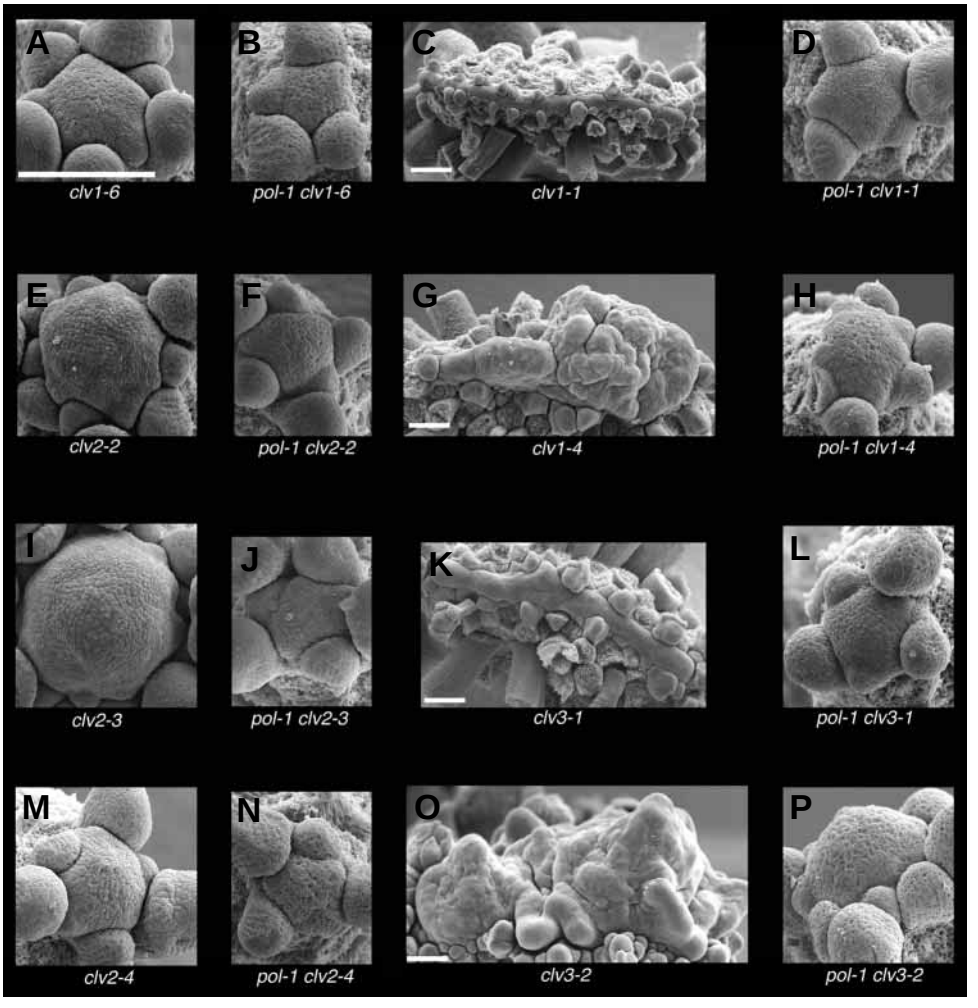

Fig. 4. *pol* suppresses *clv1*, *clv2* and *clv3* shoot meristem defects. Inflorescence shoot meristems of *clv1-6* (A), *pol-1 clv1-6* (B), *clv1-1* (C), *pol-1 clv1-1* (D), *clv2-2* (E), *pol-1 clv2-2* (F), *clv1-4* (G), *pol-1 clv1-4* (H), *clv2-3* (I), *pol-1 clv2-3* (J), *clv3-1* (K), *pol-1 clv3-1* (L), *clv2-4* (M), *pol-1 clv2-4* (N), *clv3-2* (O), and *pol-1 clv3-2* (P) were analyzed by scanning electron microscopy. C,G,K,O are shown at the same magnification, as are A,B,D-F,H-J,L-N,P. Scale bar, 100 μm.

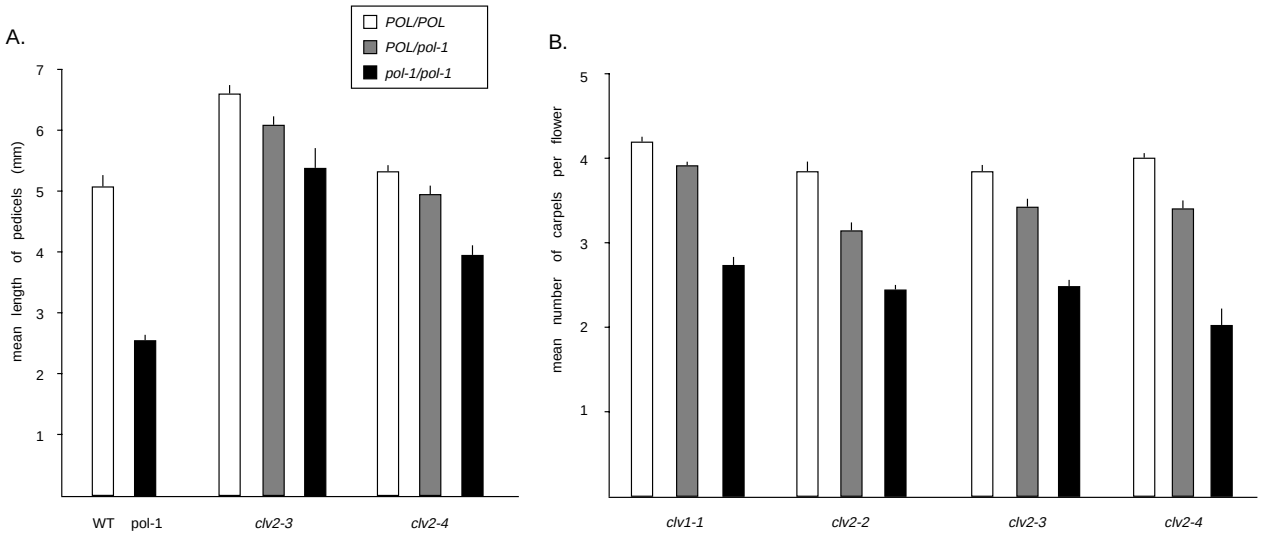

Fig. 5. *pol* dominantly suppresses both the carpel defects of *clv1* and *clv2* and the pedicel defects of *clv2*. The progeny of *clv1-1/clv1-1 pol-1/POL* and *clv2/clv2 pol-1/POL* were scored for carpel number. The *clv2* plants were also scored for pedicel length. These plants were then tested for *POL* genotype by progeny testing. (A) The mean length of pedicels (mm) for each genotype. (B) The mean number of carpels per flower. White bars represent *POL/POL*, gray bars represent *POL/pol-1*, and black bars represent *pol-1/pol-1*. At least 80 flowers and pedicels were counted for each mean calculated. Only the first ten flowers and pedicels of any given plant were analyzed. Standard errors of the mean are indicated by vertical black lines.

result of *wus* heterozygosity, we tested progeny of all fertile plants. Every plant that exhibited these phenotypes was heterozygous for *wus*, while phenotypically normal plants were largely wild-type for *WUS*, although some were *wus*/*WUS* (Table 6). Thus, *wus* semi-dominance in the *pol* mutant background was not fully penetrant.

pol enhances *stm* phenotypes

Because of the competitive nature of the interactions between the *CLV* loci and *STM* (Clark et al., 1996), we predicted that *pol* should enhance *stm* mutant phenotypes. Utilizing techniques similar to that for isolating *pol ag* plants, double mutant plants of *pol-1* with the strong and weak *stm* alleles, *stm-1* and *stm-2*, respectively, were generated (Fig. 1; Table1; see Materials and Methods). We observed that *pol* enhanced the phenotypes of both *stm* alleles. *stm-1* germinates without a shoot meristem, but is capable of developing post-embryonic leaf initiation on a portion of the mutant plants (Barton and Poethig, 1993). The formation of post-embryonic leaves was significantly reduced in *pol-1 stm-1* double mutant plants compared to *stm-1* single mutant plants (Table 7). The weak *stm-2* mutant plants initiate meristems that are eventually overtaken by organ formation, resulting in multiple rounds of meristem initiation and termination (Clark et al., 1996). *pol-1 stm-2* double mutant plants developed noticeably less post-embryonic growth compared to *stm-2* single mutant plants (Fig. 8), indicating that *pol* enhances the *stm-2* phenotype as well.

Table 5. *pol-1* reduces floral organ number in *wus-1* mutant flowers

	Average number of organs per flower*·‡		
Genotype	Sepals§	Petals¶	Stamens¶·**
pol-1 wus-1	3.0±0.1	2.4±0.1	0.6±0.1
wus-1	3.5±0.1	3.6±0.1	1.4±0.1

*Values represent the mean number ± standard error of the mean of indicated organs.

‡61 flowers were counted for each mean and standard error of the mean calculated.

§$0.2>P>0.1$.

¶$P<0.005$.

**Whorl 3 organs that developed as filamentous organs were included in the count of stamens.

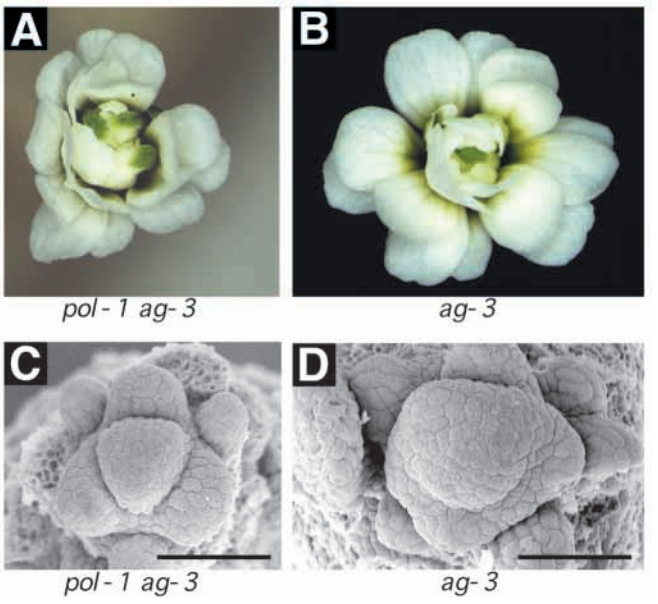

Fig. 6. *pol* specifically suppresses the indeterminacy of *clv* flowers. Flowers of *pol-1 ag-3* plants (A,C) are identical to those of *ag-3* plants (B,D) both in terms of overall morphology and in terms of floral meristem structure as analyzed by scanning electron microscopy. Scale bars, 20 μm.

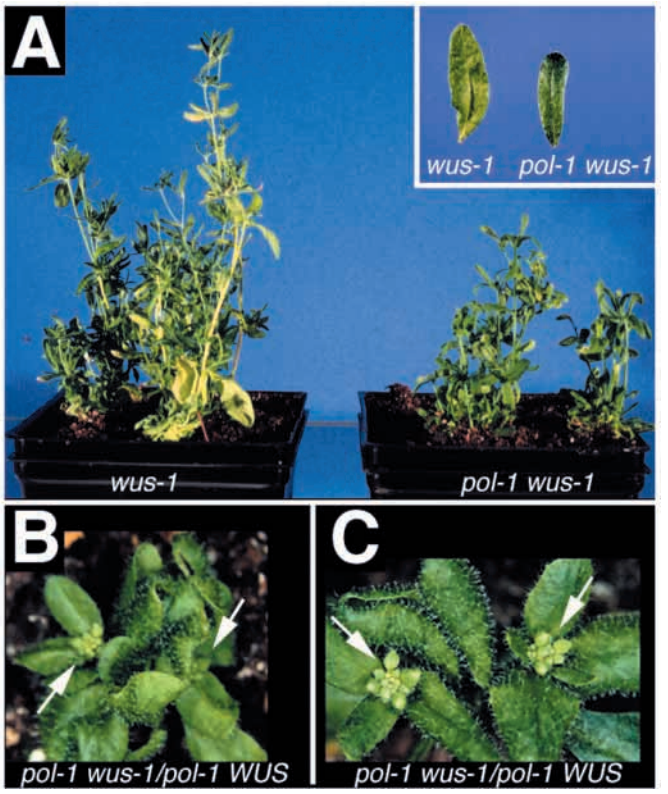

Fig. 7. *wus-1* is semi-dominant in a *pol-1* homozygous background. *wus-1* and *pol-1 wus-1* plants and leaves at 70 days (A) and *pol-1 wus-1/pol-1 WUS* plants where the primary shoot meristem terminated leading to the development of multiple lateral shoot meristems indicated by white arrows (B,C).

pol does not affect CLV1 receptor-kinase activation

The observations that *pol* dominantly suppresses the phenotypes of many *clv* alleles, and that it suppresses the plants homozygous for the null *clv3-2* allele, suggests that *POL* functions downstream of the *CLV* loci (see Discussion). The *CLV* loci all appear to act as signaling molecules at the cell surface, with CLV3 activating the membrane-localized CLV1 and CLV2 (Trotochaud et al., 1999). CLV1 activity can be assayed by examining CLV1 complex formation in vivo. CLV1 is found in two complexes: an inactive 185 kDa complex (fractions 17-18) that may be a CLV1-CLV2 heterodimer, and an active 450 kDa complex (fractions 12-13) that includes the protein phosphatase KAPP and a Rho GTPase-related protein

Table 6. *wus-1* is semi-dominant in a *pol-1* background

		Number of plants of indicated genotype					
		WUS/WUS		*wus-1/WUS*		*wus-1/wus-1*	
Phenotype	No. plants	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
Wild type	32	26	10.7	6	21.3	0	0
wus-like rosette with wild-type inflorescence	24	0	8	24	16	0	0

Progeny of a *pol-1 wus-1/pol-1 WUS* plant were scored as having wild-type phenotype, *wus*-like rosette with wild-type inflorescence phenotype, or *wus*-like rosette and inflorescence phenotype. The number of plants segregating *wus-1* was compared with the number that would be expected to segregate *wus-1*, if *wus-1*/*WUS* had no effect on the phenotype of these plants.

Table 7. *pol-1* reduces the rate of postembryonic development in *stm-1* mutants

	Rate of postembryonic development*,‡	
Days after germination	*stm-1*	*pol-1 stm-1*
15	0	0
24	0.29	0.14
27	0.39	0.21
30	0.48	0.27

*Values represent the number of plants that developed leaves in the axils of the cotyledons per total number of surviving mutant plants.
‡More than 129 viable mutant plants were counted for each rate calculated.

(Trotochaud et al., 1999). In *clv3* mutant plants, CLV1 is found exclusively in the inactive 185 kDa complex, indicating that the formation of the active complex requires CLV3.

If POL functions downstream of CLV1, then *pol* mutations should have no direct effect on CLV1 complex formation. A rigorous way to test this idea would be to examine CLV1 complex formation in *pol-1 clv3-2* double mutant plants. If *pol* suppression is downstream of CLV1, the *pol-1 clv3-2* plants should accumulate no active CLV1. On the other hand, if *pol* suppression acts by increasing activation of CLV1, then CLV1 in the active 450-kD complex may be observed in the *pol-1 clv3-2* double mutant plants. When CLV1 complex formation was assayed in *clv3-2* and *pol-1 clv3-2* plants, no difference was observed (Fig. 9). In other words, all of the CLV1 remained in the inactive 185 kDa complex despite the *pol* mutation.

DISCUSSION

Research into meristem development in *Arabidopsis* has only uncovered a handful of specific regulators. However, even in plants with the most severe mutant alleles of these regulators and in various double mutants, organ formation still occurs. This suggests that additional genes are present that allow for organ formation even in the absence of these genes. The additional genes may have remained unidentified for several reasons. First, the additional genes may regulate multiple development processes leading to pleiotropic mutant

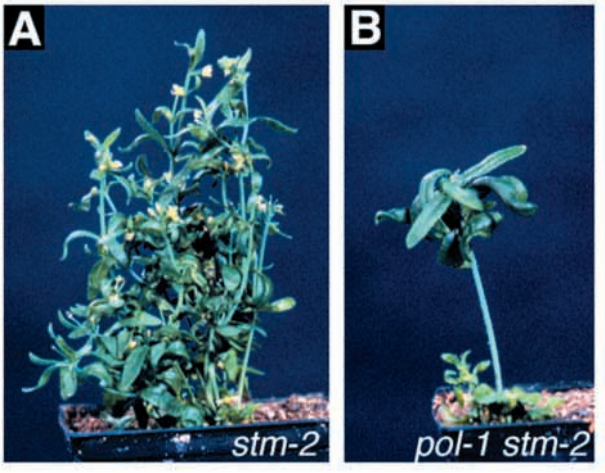

Fig. 8. *pol* enhances *stm* phenotypes. Representative *stm-2* (A) and *pol-1 stm-2* (B) plants at 70 days.

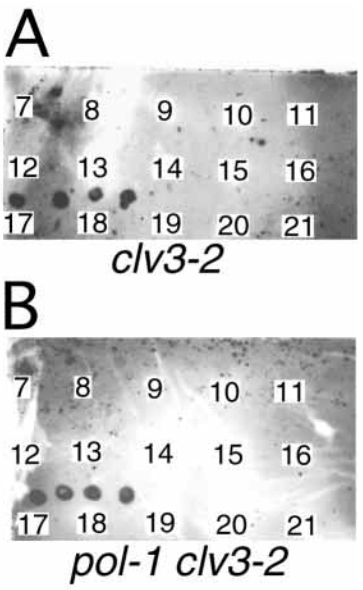

Fig. 9. *pol-1* suppression of *clv3* phenotypes is not due to altered CLV1 receptor activation. Proteins from *clv3-2* (A) and *pol-1 clv3-2* (B) plants were fractionated by gel filtration on a superose 6 column as described by Trotochaud et al. (1999). Fractions were dotted onto nitrocellulose and probed with anti-CLV1 antibodies.

phenotypes. In fact, a number of mutations with pleiotropic effects alter the development of the shoot and/or floral meristem including *revoluta*, *mgoun1*, *mgoun2*, *fasciata1*, *fasciata2*, *forever young*, and *tousled* (Callos et al., 1994; Laufs et al., 1998; Leyser and Furner, 1992; Roe et al., 1993; Talbert et al., 1995). Second, these genes may play vital roles and are lethal when mutated. Another category may be genes that carry out redundant functions, such that single mutants have no phenotypes. There is precedence for this, such as the redundant function of *AP1* and *CAL* in the regulation of floral meristem identity (Bowman et al., 1993), and *CUC1* and *CUC2* in the regulation of embryonic cotyledon and meristem formation (Aida et al., 1997). In the case of *AP1* and *CAL*, *ap1* mutants exhibit mutant phenotypes, while *cal* mutants are indistinguishable from wild type. We have characterized mutant alleles of a novel locus, *POL*, that appears to carry out a redundant function with *WUS* in much the same manner that *CAL* and *AP1* have redundant functions. Genetic interactions indicate that *POL* is a critical regulator of meristem development.

Function of *POL*

A critical question is whether *POL* functions downstream of the *CLV* loci, or in an independent pathway. The observation that *pol* suppresses the phenotypes of all *clv* alleles (even null alleles) is consistent with both hypotheses. However, several additional lines of evidence combine to provide strong support for the idea that *POL* functions downstream of the *CLV* loci. (1) *pol* suppression of the many *clv* alleles is semi-dominant. This indicates that the clv phenotypes are sensitive to the level of *POL* activity. In other words, *POL* is required in part for the accumulation of undifferentiated cells observed in *clv* mutants. Even a presumed 50% reduction of *POL* activity partially suppressed the clv phenotypes, indicating a close association of the activity of the POL and CLV gene products. (2) *pol* suppression of flower meristem determinacy is specific to the *clv* mutations. Both *clv* and *ag* plants develop indeterminate flower meristems. The indeterminacy is not altered in *pol ag* double mutant plants, while it is suppressed in *pol clv* double mutant plants. (3) The normally recessive *wus* mutation, which is known to be epistatic to *clv* mutations (Laux et al., 1996), becomes semi-dominant in a *pol* mutant background. This demonstrates that *pol* mutant plants require a full level of *WUS* activity to develop in a largely normal manner, indicating that POL and WUS function closely together in the regulation of meristem development. (4) *pol* enhances the *wus-1* phenotype, suggesting either that *POL* functions redundantly with *WUS*, or that *wus-1* is not a null allele. *wus-1* contains a nonsense mutation downstream of the coding sequence for the homeodomain, but displays phenotypes similar to alleles with nonsense mutations near the very beginning of the coding sequence, suggesting that *wus-1* is a null allele (Mayer et al., 1998). *pol wus* double mutants also exhibit synergistic phenotypes, suggesting again that these genes are redundant. Thus, the specificity for suppressing *clv* and the extensive dominant interactions are all consistent with *POL* acting within the *CLV* pathway. However, genetic analysis can never constitute proof that genes function in the same pathway – this must await a biochemical analysis of the respective gene products.

The interpretations of *POL* function should hold whether the *pol* alleles are partial or complete loss-of-function mutations. *pol* alleles are very unlikely to be gain-of-function alleles because of the relatively high number of independent *pol* alleles (three) that were isolated from a relatively small mutagenized population (Pogany et al., 1998).

Evidence for the role of *WUS*

The indication that *POL* and *WUS* function redundantly clarifies the role of *WUS* in meristem development. The previously demonstrated epistasis of *wus* mutations over *clv1* mutations (Laux et al., 1996) left open two possible roles for *WUS* in meristem development. *WUS* could function either upstream of the *CLV* loci to pattern the meristem upon which these genes act, or downstream as a target for CLV signaling. Previous genetic and molecular genetic analyses have failed to distinguish between these possibilities. The uncertainty arises from the fact the *wus* mutants do not develop functional meristems. Thus, it was unclear if the *CLV* loci are ever active in *wus* mutants. The evidence that *POL* functions redundantly with *WUS* indicates that *WUS* functions downstream of the *CLV* loci. This is because even though *pol* suppresses the phenotype of the putative null *clv3-2* allele and is epistatic to weak *clv* mutations, these results are unlikely to be the result of *POL* functioning upstream, as *pol* mutants generate functional meristems that clearly require the *CLV* loci.

stm interactions

It has been hypothesized that *STM* plays separate roles in meristem initiation and meristem maintenance (Barton and Poethig, 1993; Long et al., 1996). We have hypothesized that, in its role in meristem maintenance, *STM* positively regulates *WUS* (Clark and Schiefelbein, 1997). *stm wus* double mutant phenotypes (Endrizzi et al., 1996) are consistent with this hypothesis, but also fit with several other hypotheses as well. The model we have put forward would predict that *pol* mutations should enhance *stm* phenotypes, as *stm* plants would lack proper *WUS* activation. This is indeed what we observed – that *pol* enhances both weak and strong *stm* mutant phenotypes in a recessive manner. We did not observe dominant interactions between *stm* and *pol* mutations, nor did we observe synergistic

phenotypes in *pol stm* plants. This evidence supports the idea that *POL* is redundant with *WUS*, but not *STM*.

POL regulates pedicel length

One of the remarkable aspects of the *pol* phenotype is that it suppresses both the meristem defects and pedicel defects of *clv2* mutant plants. We have previously demonstrated that *CLV2* regulates pedicel length independent of its regulation of the floral meristem, and independent of *CLV1* and *CLV3* (Kayes and Clark, 1998). An attractive model is that *POL* functions downstream of *CLV2* in both the regulation of meristem development and the regulation of pedicel length. If this is the case, then *POL* is again redundant with a separate factor, because *CLV2* stills functions to repress pedicel length in the *pol* mutant background (i.e., *pol clv2* plants developed longer pedicels than *pol* plants). An alternative hypothesis is that *POL* functions in an independent pathway to regulate pedicel development, and that the *pol clv2* double mutant phenotype can be viewed as additive.

POL function

The likely function of *POL* is to promote the undifferentiated state of cells at the center of the shoot and floral meristems. This would be consistent with the reduction in the number of undifferentiated cells in *pol clv* double mutant plants, as well as the reduction in adventitious meristem activity in *pol wus* and *pol stm* double mutant plants. If *POL* functions downstream of the *CLV* loci as indicated by the present study, then it may be a target for CLV1 signal transduction. *CLV1* encodes a receptor-like kinase that is hypothesized to relay positional information within the meristem (Clark et al., 1997). CLV1 is activated by CLV3 (Trotochaud et al., 1999), which codes for a putative proteinaceous ligand (Fletcher et al., 1999). The active CLV1 is associated with a Rho GTPase-related protein (Trotochaud et al., 1999) which may then relay a signal that targets both POL and WUS. The genetic models of *POL* function make further predictions about the molecular nature of the POL gene product. The proposed redundant nature of *POL* and *WUS* function would suggest that because *WUS* encodes a putative transcription factor, *POL* may code either for a transcription factor or a protein that regulates one. *POL* should be expressed both in the shoot meristem in an overlapping domain with *WUS*, and in the developing floral pedicel. Because *POL* function in the shoot meristem is not readily detectable, we cannot rule out the possibility that *POL* functions in many developmental processes, but that phenotypes in these processes are also not readily apparent. Resolution of these ideas await the identification of the *POL* gene.

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REFERENCES

Aida, M., Ishida, T., Fukaki, H., Fujisawa, H. and Tasaka, M. (1997). Genes involved in organ separation in Arabidopsis – an analysis of the cup-shaped cotyledon mutant. *Plant Cell* **9**, 841-857.

Barton, M. K. and Poethig, R. S. (1993). Formation of the shoot apical meristem in *Arabidopsis thaliana*: An analysis of development in the wild type and *shoot meristemless* mutant. *Development* **119**, 823-831.

Bowman, J. L., Alvarez, J., Weigel, D., Meyerowitz, E. M. and Smyth, D. R. (1993). Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. *Development* **119**, 721-743.

Bowman, J. L., Smyth, D. R. and Meyerowitz, E. M. (1989). Genes directing flower development in *Arabidopsis. Plant Cell* **1**, 37-52.

Callos, J. D., Dirado, M., Xu, B. B., Behringer, F. J., Link, B. M. and Medford, J. I. (1994). The *FOREVER YOUNG* gene encodes an oxidoreductase required for proper development of the *Arabidopsis* vegetative shoot apex. *Plant J.* **6**, 835-847.

Clark, S. E. (1997). Organ formation at the vegetative shoot meristem. *Plant Cell* **9**, 1067-1076.

Clark, S. E., Jacobsen, S. E., Levin, J. and Meyerowitz, E. M. (1996). The *CLAVATA* and *SHOOT MERISTEMLESS* loci competitively regulate meristem activity in *Arabidopsis*. *Development* **122**, 1567-1575.

Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1993). *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. *Development* **119**, 397-418.

Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1995). *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. *Development* **121**, 2057-2067.

Clark, S. E. and Schiefelbein, J. W. (1997). Expanding insights into the role of cell proliferation in plant development. *Trends Cell Biol.* **7**, 454-458.

Clark, S. E., Williams, R. W. and Meyerowitz, E. M. (1997). The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* **89**, 575-585.

Endrizzi, K., Moussain, B., Haecker, A., Levin, J. Z. and Laux, T. (1996). The *SHOOT MERISTEMLESS* gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than the meristem genes *WUSCHEL* and *ZWILLE*. *Plant J.* **10**, 967-979.

Fletcher, J. C., Brand, U., Running, M. P., Simon, R. and Meyerowitz, E. M. (1999). Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. *Science* **283**, 1911-1914.

Jeong, S., Trotochaud, A. E. and Clark, S. E. (1999). The Arabidopsis *CLAVATA2* gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *Plant Cell* **11**, 1925-1933.

Kayes, J. M. and Clark, S. E. (1998). *CLAVATA2*, a regulator of meristem and organ development in Arabidopsis. *Development* **125**, 3843-3851.

Laufs, P., Dockx, J., Kronenberger, J. and Traas, J. (1998). *MGOUN1* and *MGOUN2* – 2 genes required for primordium initiation at the shoot apical and floral meristems in *Arabidopsis thaliana*. *Development* **125**, 1253-1260.

Laux, T., Mayer, K. F. X., Berger, J. and Jurgens, G. (1996). The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis*. *Development* **122**, 87-96.

Leyser, H. M. O. and Furner, I. J. (1992). Characterization of three shoot apical meristem mutants of *Arabidopsis thaliana*. *Development* **116**, 397-403.

Long, J. A., Moan, E. I., Medford, J. I. and Barton, M. K. (1996). A member of the *KNOTTED* class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis*. *Nature* **379**, 66-69.

Mayer, K. L., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G. and Laux, T. (1998). Role of *WUSCHEL* in regulating stem cell fate in the Arabidopsis shoot meristem. *Cell* **95**, 805-815.

Pogany, J. A., Simon, E. J., Katzman, R. B., de Guzman, B. M., Yu, L. P., Trotochaud, A. E. and Clark, S. E. (1998). Identifying novel regulators of shoot meristem development. *J. Plant Res.* **111**, 307-313.

Roe, J. L., Rivin, C. J., Sessions, R. A., Feldmann, K. A. and Zambryski, P. C. (1993). The *TOUSLED* gene in *A. thaliana* encodes a protein kinase homolog that is required for leaf and flower development. *Cell* **75**, 939-950.

Smyth, D. R., Bowman, J. L. and Meyerowitz, E. M. (1990). Early flower development in *Arabidopsis. Plant Cell* **2**, 755-767.

Talbert, P. B., Alder, H. T., Parks, D. W. and Comai, L. (1995). The *REVOLUTA* gene is necessary for apical meristem development and for limiting cell divisions in the leaves and stems of *Arabidopsis thaliana*. *Development* **121**, 2723-2735.

Trotochaud, A. E., Hao, T., Guang, W., Yang, Z. and Clark, S. E. (1999). The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. *Plant Cell* **11**, 393-405.