APETALA2 Regulates the Stem Cell Niche in the *Arabidopsis* Shoot Meristem [W]

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Postembryonic organ formation in higher plants relies on the activity of stem cell niches in shoot and root meristems where differentiation of the resident cells is repressed by signals from surrounding cells. We searched for mutations affecting stem cell maintenance and isolated the semidominant *l28* mutant, which displays premature termination of the shoot meristem and differentiation of the stem cells. Allele competition experiments suggest that *l28* is a dominant-negative allele of the *APETALA2* (*AP2*) gene, which previously has been implicated in floral patterning and seed development. Expression of both *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*) genes, which regulate stem cell maintenance in the wild type, were disrupted in *l28* shoot apices from early stages on. Unlike in floral patterning, *AP2* mRNA is active in the center of the shoot meristem and acts via a mechanism independent of *AGAMOUS*, which is a repressor of *WUS* and stem cell maintenance in the floral meristem. Genetic analysis shows that termination of the primary shoot meristem in *l28* mutants requires an active *CLV* signaling pathway, indicating that *AP2* functions in stem cell maintenance by modifying the *WUS-CLV3* feedback loop.

INTRODUCTION

Postembryonic organ formation in plants requires the maintenance of small stem cell pools in the shoot, floral, and root meristems (Weigel and Jürgens, 2002; Laux, 2003). Similar to animal stem cell systems, the meristems provide a microenvironment, the stem cell niche, in which differentiation of the resident cells is repressed, whereas daughter cells that leave this site undergo differentiation. In the *Arabidopsis thaliana* shoot meristem, the stem cells are located in the three outermost cell layers of the most central region of the shoot apex. The stem cells cannot be distinguished by their appearance from the surrounding meristem cells, and stem cell function can only be unambiguously identified by clonal studies (Stewart and Dermen, 1970). Expression of *CLAVATA3* (*CLV3*) mRNA (Fletcher et al., 1999) that coincides with the presumed position of the apical stem cells provides an operational marker for stem cell identity in the shoot meristem.

Only a few mutants have been isolated that specifically fail to protect shoot meristem stem cells from differentiation. One of the genes identified from mutant studies encodes the homeodomain protein WUSCHEL (WUS). *WUS* is expressed in a domain underneath the stem cells termed the organizing center (OC) and is required and sufficient to maintain the overlying stem cells

undifferentiated (Mayer et al., 1998; Schoof et al., 2000). The stem cells in turn signal back via the CLV3 peptide to restrict the size of the OC, thereby creating a negative feedback loop to dynamically control the size of the stem cell population (Brand et al., 2000; Schoof et al., 2000). CLV3 presumably acts as an extracellular ligand of the CLV1 receptor kinase complex, which eventually leads to downregulation of *WUS* transcription in the recipient cells (Clark et al., 1997; Stone et al., 1998; Trotochaud et al., 1999; Rojo et al., 2002; Lenhard and Laux, 2003). In floral meristems that are homologous to indeterminate shoot meristems, the *AGAMOUS* (*AG*) gene represses *WUS* expression late in flower development to terminate the stem cell niche after a limited number of organs have been formed (Lenhard et al., 2001; Lohmann et al., 2001).

The primary shoot meristem is formed during embryo development (Barton and Poethig, 1993). In *Arabidopsis*, the onset of *WUS* expression in precursor cells of the OC in the 16-cell embryo suggests that shoot meristem formation has been initiated already at this stage (Mayer et al., 1998). *CLV3* expression is detected from late heart stage on between the outgrowing cotyledons (Fletcher et al., 1999). In bent cotyledon embryos, the primary shoot meristem becomes visible as a bulge of small cytoplasmic cells that after germination give rise to a rosette of leaves before an inflorescence stem is formed, carrying cauline leaves with axillary shoot meristems and bractless floral meristems.

Many aspects of the mechanisms that operate in the stem cell niches to maintain stem cells undifferentiated are not understood and might have escaped genetic analysis due to genetic redundancy. Therefore, we performed an extensive genetic screen to detect presumably rare dominant-negative mutations affecting stem cell maintenance. Here, we report the isolation and functional characterization of the semidominant *l28* mutant that in the homozygous state is unable to keep stem cells undifferentiated similar to the *wus* mutant. We show that

the premature termination of the shoot meristem in *l28* mutants is caused by a mutation in the *APETALA2* (*AP2*) gene that was previously identified as one of the components of the ABC model in floral patterning, where it represses *AG* (Bowman et al., 1991; Drews et al., 1991), in floral transition (Jofuku et al., 1994; Okamuro et al., 1997b), and in the control of seed size (Jofuku et al., 2005; Ohto et al., 2005).

In this article, we show that maintenance of the stem cell niche in the primary shoot meristem requires the activity of *AP2* and redundant factor(s) via an *AG* independent mechanism that involves interaction with the *WUS-CLV3* feedback loop.

RESULTS

The *l28* Mutation Affects Shoot Apical Meristem Maintenance

The *l28* mutant was identified in an ethyl methanesulfonate mutagenesis screen for *Arabidopsis* seedlings defective in shoot meristem development. In wild-type seedlings (Figure 1A), the shoot meristem successively forms a rosette of on average eight leaves and an inflorescence shoot carrying two to three cauline leaves with axillary side branches, and many flowers. By contrast,
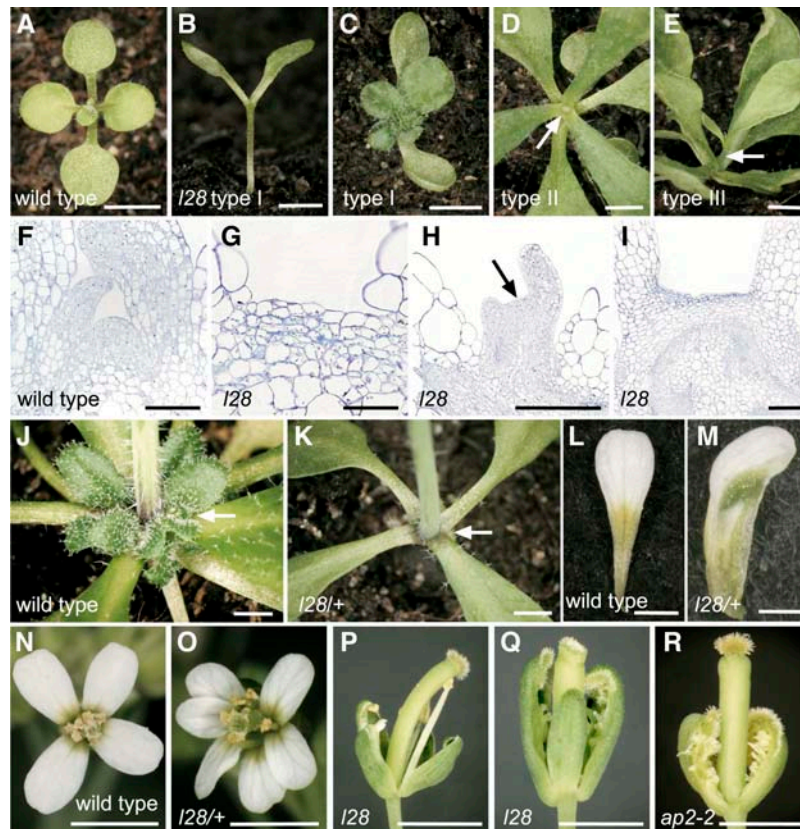

Figure 1. Phenotypes of *l28* Homozygous and Heterozygous Mutants.

(A) In 12-d-old wild-type seedlings, the shoot meristem has initiated rosette leaves.

(B) A 12-d-old *l28* mutant in which the shoot meristem has terminated prematurely.

(C) A 20-d-old *l28* seedling that has formed a few leaves from a transiently active adventitious meristem.

(D) and **(E)** The 24-d-old *l28* plants that terminated shoot meristem activity after six leaves (**[D]**; arrow indicates terminated apex) or that terminated after forming a short stem (**[E]**; arrow).

(F) to **(I)** Histological sections stained with toluidine blue.

(F) In a 12-d-old wild-type seedling, the shoot meristem is characterized by small and densely stained cells.

(G) In a 12-d-old *l28* mutant, the apical cells are larger and more vacuolated than in the wild type.

(H) In a 16-d-old *l28* mutant, an adventitious meristem has been formed and already terminated (arrow).

(I) A 24-d-old *l28* mutant. The cells of the enlarged flat apex have undergone differentiation.

(J) and **(K)** The axillary inflorescences that are initiated in the axils of the rosette leaves (arrows) in wild-type plants **(J)** are absent in *l28* heterozygous plants **(K)**.

(L) and **(M)** Second whorl petal of a wild-type flower **(L)** and chimeric petal **(M)** with patches of sepalloid green tissue of a *l28/+* flower.

(N) to **(R)** Flowers from the wild type **(N)**, *l28/+* **(O)**, homozygous *l28* mutants (**[P]** and **[Q]**), and a flower from the *ap2-2* mutant **(R)**.

Bars = 5.0 mm in **(A)** to **(E)**, **(J)**, and **(K)**, 50 μm in **(F)** to **(I)**, and 2.0 mm in **(L)** to **(R)**.

homozygous *l28* mutant seedlings (genotyped by PCR after cloning of the gene; see below) displayed premature termination of the shoot meristem in an empty flat apex. We categorized the seedling phenotypes into three classes depending on the developmental stage when meristem termination occurred (Table 1).

Type I seedlings (12%) showed the most severe phenotype with only two cotyledons but no shoot meristem at 12 d after germination (Figure 1B). Approximately 1 week later, a number of leaves would form (Figure 1C) before a flat apex appeared. This suggests that these plants do not form a functional primary (embryo derived) shoot meristem, whereas they are able to give rise to transiently active adventitious meristems. Inflorescences, however, were never formed. Type II seedlings (73%) gave rise to between one and eight rosette leaves indistinguishable from wild-type plants before the shoot meristem terminated in a flat apex (Figure 1D, arrow). Type III seedlings (16%) gave rise to a rosette of leaves and a short inflorescence stem before meristem termination occurred (Figure 1E, arrow). The inflorescence stem carried a few cauline leaves but, with rare exceptions (see below), no flowers. Unlike the shoot meristem, the root meristem of homozygous *l28* mutants of all three types appeared normal (data not shown).

In order to reveal the cellular basis of the premature meristem termination, we studied histological sections of homozygous *l28* mutants. In wild-type plants, the shoot meristem can be recognized as a convex structure that is organized in distinct layers and contains small, cytoplasmically rich cells (Figure 1F). By contrast, terminated apices of homozygous *l28* plants were flat and contained larger and more vacuolated cells when compared with the wild type, suggesting that they have undergone differentiation (Figures 1G to 1I). Notably, this phenotype was indistinguishable from the *wus-1* mutant (Laux et al., 1996), which is defective in stem cell maintenance. Complementation analysis demonstrated that *l28* was not allelic to *wus-1* (data not shown), indicating that it defined a novel genetic locus involved in stem cell regulation.

In contrast with homozygotes, heterozygous *l28/+* plants had an indeterminate shoot meristem and gave rise to flowering shoots. The number of secondary meristems in the axils of the rosette leaves, however, was reduced in comparison with the wild type (Figures 1J and 1K; Table 2). Only in a scarce minority of *l28/+* plants (2/712) did the shoot meristem terminate prematurely in a flat apex similar to homozygous mutants. Thus, shoot

meristem development was affected by the *l28* mutation in a semidominant manner.

In addition to the shoot meristem, flower development was also affected by the *l28* mutation. Wild-type flowers consist of four whorls of organs from outermost to inner: four sepals, four petals, six stamens, and a gynoecium of two fused carpels (Figure 1N). Only three out of 800 homozygous *l28* mutants formed flowering inflorescences, allowing us to study mutant flower development. The first five flowers formed on each *l28* inflorescence had reduced organ numbers in the second and third whorls (Figure 1P). In later formed flowers, organs in whorls two and three were completely absent, and carpelloid organs instead of sepals were present in the first whorl (Figure 1Q). The few inflorescences that did not form any axillary meristems eventually terminated in a mass of carpels (data not shown). Notably, the floral defects and the increasing severity during inflorescence development resembled those of strong *ap2* mutants (Figure 1R) (Bowman et al., 1989, 1991).

Flowers of *l28/+* plants displayed weaker defects: namely, variable organ numbers, an increased number of petals (Figure 1O), and chimeric organs in whorls two and three (Figure 1M). In addition, the gynoecia elongated prematurely and protruded from the flower bud (data not shown).

Taken together, the *l28* mutation affects two separate aspects of plant development: maintenance of undifferentiated cells in the shoot meristem and floral patterning. In this article, we focus on the function of *l28* in shoot meristem maintenance.

Positional Cloning of *l28*

The *l28* mutation was mapped to a 9.3-kb interval on chromosome 4 between derived cleaved-amplified polymorphic sequence (dCAPS) markers TWC11 and TWC7 (Figure 2A; see Methods for details). This region contained the promoter and most of the coding sequence of the floral organ identity gene *APETALA2* (*AP2*). We therefore compared the genomic sequences of *AP2* in the wild type and in *l28* and identified a single-base exchange in the coding sequence of *l28* (Figure 2B). *AP2* encodes one of the founding members of the large AP2/ERF family of putative transcription factors that are marked by the presence of at least one AP2 domain, which is presumably involved in DNA binding (Okamuro et al., 1997a; Allen et al., 1998; Nole-Wilson and Krizek, 2000; Magnani et al., 2004; Nole-Wilson et al., 2005). Within this

Table 1. *l28* Gene Dosage Effects on Primary Shoot Meristem Termination

F1 from Cross	*n*	Distribution of Phenotypes			Type I: Early Stop	Type II: Shoot Meristem Termination after *n* Leaves (%)								Type III: Late Stop
		WT (%)[a]	FP (%)[b]	Stop (%)[c]		1	2	3	4	5	6	7	8	
l28/+ × wild type	1418	49.8	50.1	0.1					50					50
l28/+ × *l28/+*	3260	25.9	49.8	24.3	12	4	4	2	13	18	22	9	1	16
l28/+ × *ap2-1*	2154	49.1	44.1	6.8					8	35	31	10	0	17
l28/+ × *ap2-2*	2163	50.6	8.8	40.6	7	1	2	2	23	25	21	3		16

[a] WT, indistinguishable from the wild type.
[b] FP, floral phenotype indistinguishable from *l28* heterozygous plants.
[c] Stop, shoot meristem termination at the seedling stage.

Table 2. *l28* Gene Dosage Effects on Rosette Leaves and Axillary Inflorescences

Genotype	*n*	Rosette Leaves	Axillary Inflorescences
AP2/AP2/AP2	30	7.7 ± 0.6	4.1 ± 0.5
l28/AP2/AP2	32	6.3 ± 0.6	1.3 ± 0.8
AP2/AP2	37	7.5 ± 0.7	4.6 ± 0.5
l28/AP2	50	5.3 ± 0.7	1.3 ± 0.7
l28/ap2-1	52	4.6 ± 0.6	0.9 ± 0.7
l28/ap2-2	70	4.5 ± 0.8	0.3 ± 0.4
ap2-1/ap2-1	30	7.2 ± 0.6	4.2 ± 0.5
ap2-2/ap2-2	35	7.1 ± 0.6	4.0 ± 0.4
clv3-2/clv3-2	18	7.9 ± 0.7	4.7 ± 0.8
l28/+/clv3-2/clv3-2	19	7.7 ± 0.7	0.3 ± 0.5
l28/l28 clv3-2/clv3-2	10	6.9 ± 1.0	0.0 ± 0.0

family, AP2 defines a subgroup of 15 proteins that contain two copies of the AP2 domain. The nucleotide change from G to A at position 433 in the *l28* allele causes a change of Glu residue 145 to Lys in the first of the two AP2 domains of AP2 (Figure 2B).

Transformation of a genomic DNA fragment containing the *l28* mutation into wild-type plants caused similar defects as observed in *l28/+* mutants in the T1 generation, and segregating plants in the T2 generation terminated shoot meristem development similar to homozygous *l28* mutants (see Supplemental Table 1 online). Conversely, transformation of a wild-type *AP2* genomic DNA rescued shoot meristem development in homozygous *l28* mutants (see Supplemental Table 1 online). This indicates that the identified mutation in the *AP2* gene causes the *l28* phenotype.

l28 Suggests a Novel Role of *AP2* in Stem Cell Maintenance

Previous studies of recessive loss-of-function mutants had not revealed a function of *AP2* in shoot meristem maintenance. This posed the question whether the *l28* mutation uncovers a yet unknown function of AP2 or whether the mutated protein interferes with a process not affected by the wild-type protein. To address this question, we investigated whether wild-type and mutant proteins compete for the same target(s) in allele dosage experiments.

First, we reduced wild-type *AP2* activity from *l28* heterozygous plants by crossing *l28/+* plants to homozygous *ap2-1* and *ap2-2* mutants. The *ap2-1* mutation results in an amino acid exchange and phenotypically is a weak loss-of-function allele, whereas the *ap2-2* mutation renders no detectable protein and is a putative null allele (Bowman et al., 1989; Chen, 2004). The frequency of primary shoot meristem termination increased dramatically with decreasing AP2 activity from 0.3% in *l28/AP2* plants to 13.4% in *l28/ap2-1* plants and to 82.3% in *l28/ap2-2* plants (Figure 2C; plants genotyped by PCR). In addition, the average number of leaves formed before the shoot meristem terminated declined with reduction of AP2 activity (Table 1), indicating that the more the *l28* allele is in excess of wild-type *AP2*, the earlier in development the primary shoot meristem terminates. Note that the frequency of meristem termination in *l28/ap2-2* plants was still significantly lower than that of *l28* homozygotes (99.7%). This

indicates that the l28 protein acts in a dosage-dependent manner in the absence of the wild-type AP2 protein, suggesting competition between l28 and factor(s) redundant to AP2.

The remaining fraction of plants within each genotype that did not terminate the shoot meristem resembled *l28/+* plants and displayed decreasing axillary meristem formation the more AP2 activity was reduced (Table 2).

In a complementary experiment, we increased the number of wild-type *AP2* gene copies by crossing *l28/AP2* plants to a tetraploid *Arabidopsis* line to generate triploid F1 plants. *l28/AP2/AP2* plants showed a significantly weaker phenotype than *l28/AP2* plants in that they formed more rosette leaves (Table 2). In addition, transformation of the wild-type *AP2* gene into *l28/+* plants also alleviated the mutant defects (data not shown).

Together, these results indicate that the *l28* phenotype is enhanced by reduction and alleviated by increase of AP2 wild-type activity in a dosage-dependent manner, suggesting that l28 and AP2 proteins compete for the same target or an interacting protein.

Shoot Meristem Size Is Reduced in *l28*

Since the *l28* mutation interfered with wild-type *AP2* activity, we carefully examined *ap2* loss-of-function mutants for yet undetected effects on shoot meristem development. First, we analyzed the shape and the size of the shoot meristem in mature embryos. The shape of the mutant shoot meristems was convex as in the wild type (Figures 3A and 3B). However, shoot meristem size measured by the number of epidermal cells across the apical dome was significantly reduced in *ap2-1* (9.0 cells), *ap2-2* (8.3 cells), and *l28* (8.1 cells) mature embryos in comparison with the wild type (11.2 cells; Figure 3G). This reduction in size was specific for the shoot meristem and contrasts with the enlargement of all other organs in the *ap2* embryo compared with the wild type (see Supplemental Figure 1 online; Jofuku et al., 2005; Ohto et al., 2005).

We next measured the size of inflorescence meristems by scanning electron microscopy as described previously (Lenhard and Laux, 2003). The size of the shoot meristem was reduced on average by 23% in *l28* heterozygous plants in comparison with the wild type (Figures 3C to 3F and 3H). Surprisingly, *ap2-1* and *ap2-2* mutants displayed an even more pronounced reduction of up to ~30% compared with the wild type, even though these mutants display indeterminate shoot meristem activity. By contrast, root meristem size in these mutants was the same as in the wild type, showing that this effect was specific for the shoot meristem (see Supplemental Figure 1 online).

Thus, recessive loss-of-function *ap2* mutations and the *l28* mutation both result in a size decrease of the shoot meristem at the embryonic and inflorescence stages. However, since stem cell maintenance is only affected in the *l28* mutant, the reduced meristem size per se appears not to be the cause of premature meristem termination.

AP2 Is Expressed in Immature Tissues

Since our results indicate a novel function of *AP2* in the shoot meristem as early as in embryos, we analyzed *AP2* expression throughout shoot meristem development by in situ hybridization.
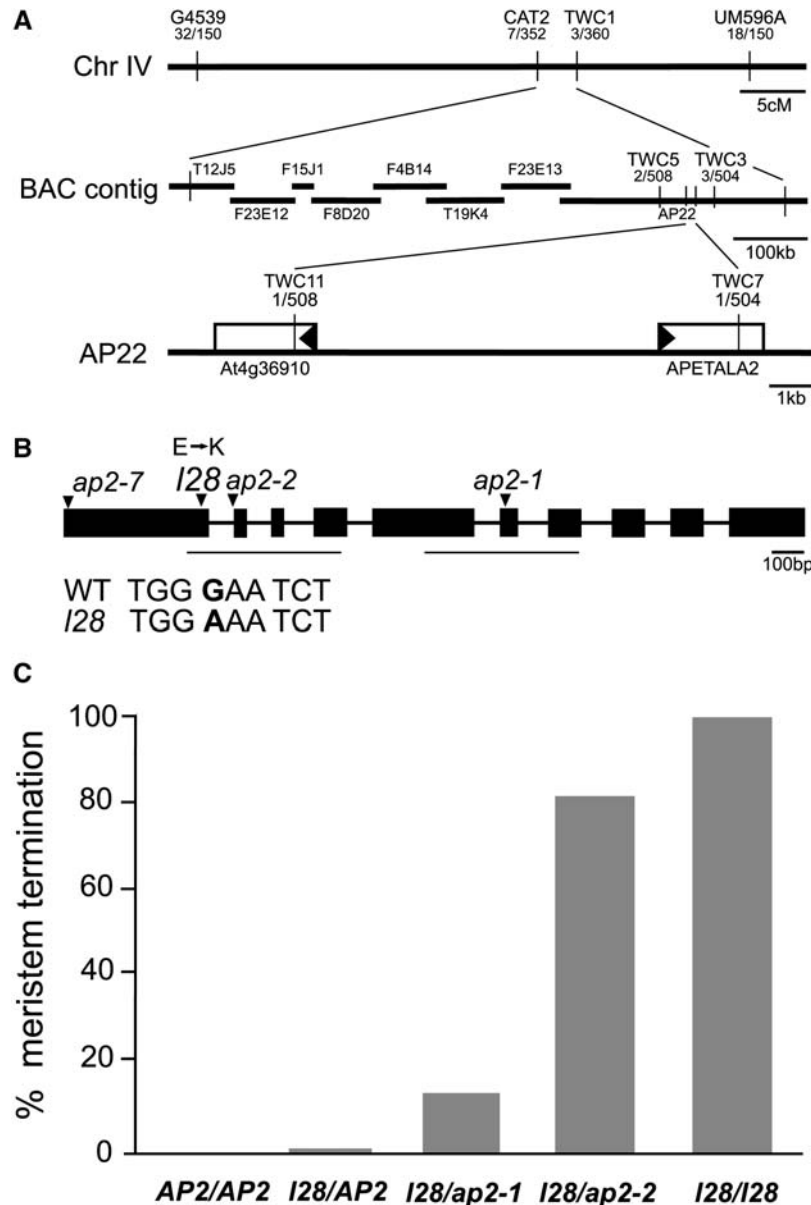
Figure 2. Cloning of *I28* and the Gene Dosage Experiment.

(A) Genetic mapping of *I28* to BAC AP22 on chromosome 4. The markers used for mapping are shown as well as the number of recombination breakpoints between *I28* and these markers. The *I28* mutation was mapped to a 9.3-kb interval that included the *AP2* gene.

(B) Genomic organization of *AP2*. The sites of the *I28*, *ap2-1*, *ap2-2*, and *ap2-7* mutations are shown. The sequences of the two AP2 domains are underlined.

(C) *I28* gene dosage experiment. The frequency of the shoot meristem termination by *I28* is enhanced by reduction of AP2 wild type activity in a dosage-dependent manner.

We detected uniform *AP2* expression throughout the embryo from the globular stage on (Figures 4A to 4C). Before this stage, no signal over background was observed (data not shown). After germination, *AP2* mRNA was expressed in inflorescence and floral meristems (Figure 4E) consistent with previous studies (Jofuku et al., 1994). Since gene expression in differentiated cells cannot easily be detected by in situ hybridization due to the small fraction of cytoplasm in the vacuolated cells, we fused 5 kb of the *AP2* upstream sequence to the β-*glucuronidase* (*GUS*) coding region. This promoter fragment provided *GUS* expression in embryos and in shoot meristems indistinguishable from the *AP2* mRNA pattern and recapitulated the *I28* phenotype when fused to the *I28* coding region (see Supplemental Table 1 online). In addition to the embryo and the shoot meristem, we detected
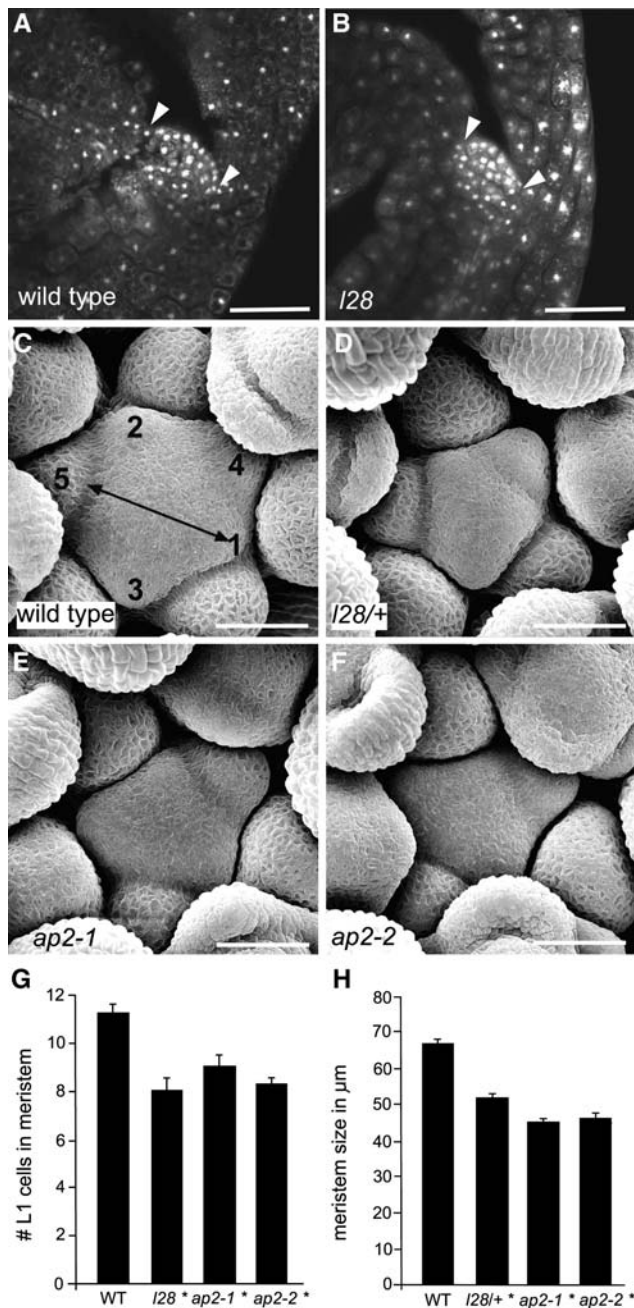
Figure 3. Meristem Size Is Reduced in *ap2* Mutants.

(A) and **(B)** Confocal laser scanning microscopy of propidium iodide–stained embryos. Wild-type **(A)** and *l28* homozygous mutant **(B)** embryos. The arrowheads indicate the size of the embryonic shoot meristem as measured by the number of L1 cells.

(C) to **(F)** Scanning electron micrographs of shoot apices. In **(C)**, young flower primordia are numbered successively from 1 to 5, and the double-headed arrow indicates the distance used to measure the size of the inflorescence meristem. Bars = 50 μm **(A)** to **(F)**.

(G) and **(H)** Average embryonic meristem size **(G)** and average inflorescence meristem size **(H)**. At least 10 plants of each genotype were analyzed. Asterisk indicates statistically significantly different from the wild type (Student's *t* test, P < 0.01).

strong *GUS* expression in leaf primordia and in young leaves, floral organs, stem, and pedicels (data not shown) but declining levels of expression as organs underwent maturation (Figure 4G).

Thus, *AP2* is strongly expressed in all immature tissues from early embryo stages on, whereas expression levels decrease as cells undergo differentiation in maturing organs.

The *l28* Mutation Represses *WUS* and *CLV3* Expression in the Shoot Meristem Stem Cell Niche

The striking similarity of *l28* and *wus* seedling phenotypes raised the question of whether *AP2* acts together with the *WUS-CLV3* pathway in stem cell regulation. To this end, we investigated the expression of *WUS* and *CLV3* in *l28* mutant embryos.

In segregating bent cotyledon embryos from an *l28/+* mother plant, we detected *WUS* expression in the subepidermal precursor cells of the OC indistinguishable from the wild type in 42% ($n = 81$) (Figure 5A), reduced expression in 48% (Figure 5B), and no expression in 10% (Figure 5C) of all embryos. Similarly, we detected *CLV3* mRNA expression at the stem cell position indistinguishable from the wild type (Figure 5D) but also embryos where *CLV3* expression was reduced to one or two cells or completely absent (Figures 5E and 5F). In this case, a *GUS* reporter gene was available (Lenhard et al., 2002) that is correctly expressed during embryo development (A. Sarkar and T. Laux, unpublished data) and thus allowed a detailed segregation analysis. The staining efficiency of dissected embryos in the wild type was 90%, and we corrected the observed frequencies accordingly. Thirty-four percent of the bent cotyledon embryos ($n = 290$) from an *l28/+* mother plant stained in a wild-type pattern (Figure 5G), 51% displayed weaker staining intensities (Figure 5H) or a reduction of the *CLV3* expression domain to one or two cells (Figure 5I), and 15% did not show *CLV3:GUS* expression at all (Figure 5J). A similar distribution was observed in torpedo stage and mature embryos (data not shown).

The segregation ratios suggests that a large part of embryos homozygous for the *l28* mutation did not express *WUS* and *CLV3* and that most heterozygous embryos expressed both genes in a reduced domain.

We then analyzed expression of *CLV3:GUS* and *WUS:GUS* reporter genes (Lenhard et al., 2002) in histological sections of 12-d-old homozygous *l28* seedlings (genotyped by PCR). At this stage, ~10% of the seedlings had not yet terminated shoot meristem activity and still displayed a convex apex, and we detected wild-type-like expression of both reporter genes (Figures 5K and 5M). By contrast, the remaining seedlings displayed a flat apex, and we did not detect *GUS* staining for either of the two reporter genes in the majority of these seedlings (Figures 5L and 5N). Occasionally we observed weak expression in seedlings with flat apices (data not shown). We assume that these represent apices that terminated just prior to tissue harvest and still showed weak staining due to the stability of the GUS protein. In situ detection of ubiquitin mRNA (*UBQ4*) as a positive control in terminated *l28* apices indicated that downregulation of *CLV3* and *WUS* expression was not due to a general transcriptional inactivity in the apex (data not shown).

Taken together, embryonic expression of both *WUS* and *CLV3* genes is reduced or abolished by the *l28* mutation apparently in
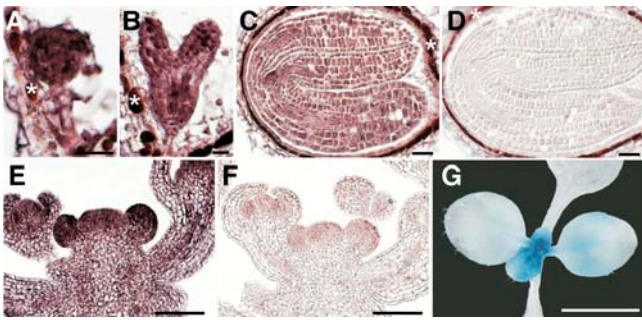
Figure 4. Expression Pattern of *AP2*.

(A) to **(F)** In situ hybridization using *AP2* antisense probe (**[A]** to **[C]** and **[E]**) and sense control (**[D]** and **[F]**). Signal is detected as reddish color. Background staining independent of the in situ procedure is marked (asterisks). Globular stage embryo **(A)**, heart stage embryo **(B)**, mature embryo (**[C]** and **[D]**), inflorescence meristems (**[E]** and **[F]**). Bars = 20 μm in **(A)** and **(B)** and 50 μm in **(C)** to **(F)**.
(G) A 14-d-old *AP2:GUS* seedling. Bar = 5.0 mm.

a dosage-dependent manner as early as at the torpedo embryo stage. Consistent with the variability of shoot meristem termination in homozygous *l28* mutants, loss of *WUS* and *CLV3* expression occurred at different times in development between individual embryos.

AP2 Functions Independently of *AG* in Shoot Meristem Stem Cell Maintenance

AP2 acts as a repressor of *AG* expression in the floral meristem (Drews et al., 1991), and *AG* represses *WUS* expression late in floral development (Lenhard et al., 2001; Lohmann et al., 2001). This raised the possibility that the repression of *WUS* and the termination of the shoot meristem in *l28* mutants were caused via ectopic expression of *AG.* To test this hypothesis, we analyzed shoot meristem development in a segregating progeny of a *l28/+ ag-1/+* mother plant (see Supplemental Table 2 online). We found that both *l28/l28 ag-1/ag-1* and *l28/l28 ag-1/+* double mutants prematurely terminated the shoot meristem in a flat apex indistinguishable from homozygous *l28* mutants, indicating that reduction of *AG* activity from homozygous *l28* mutants did not affect shoot meristem termination. Therefore, in shoot meristem stem cell maintenance, *AP2* acts in a mechanism independent of *AG*.

clv Mutations Rescue Shoot Meristem Development in *l28*

To examine whether *AP2* genetically interacts with the *WUS-CLV3* pathway in the regulation of the shoot meristem stem cell niche, we analyzed double mutant combinations. Mutations in genes of the CLV signaling pathway result in a gradual increase of the *WUS* expression domain and in turn in a progressively enlarging shoot meristem size (Clark et al., 1993, 1995). We analyzed the progeny (genotyped by PCR) of plants homozygous for the strong loss-of-function alleles *clv3-2* or *clv1-10* and heterozygous for *l28.* We obtained analogous results with both mutants and will concentrate on the results for *clv3-2.*

clv3-2 single mutants displayed a progressively enlarging shoot meristem and supernumerary floral organs, most notably stamens and carpels (Figures 6E and 6F) (Clark et al., 1995).

All *clv3-2 l28* doubly homozygous seedlings possessed an indeterminate primary shoot meristem that progressively enlarged and formed a fasciated inflorescence indistinguishable from *clv3* single mutants (Figures 6J to 6L). However, double mutants did not form any side branches in the axils of rosette or cauline leaves, similar to *l28* mutants alone (cf. Figures 6D and 6J, Table 2). Thus, the *clv3-2* mutation suppressed the premature termination of the embryonically formed primary shoot meristem
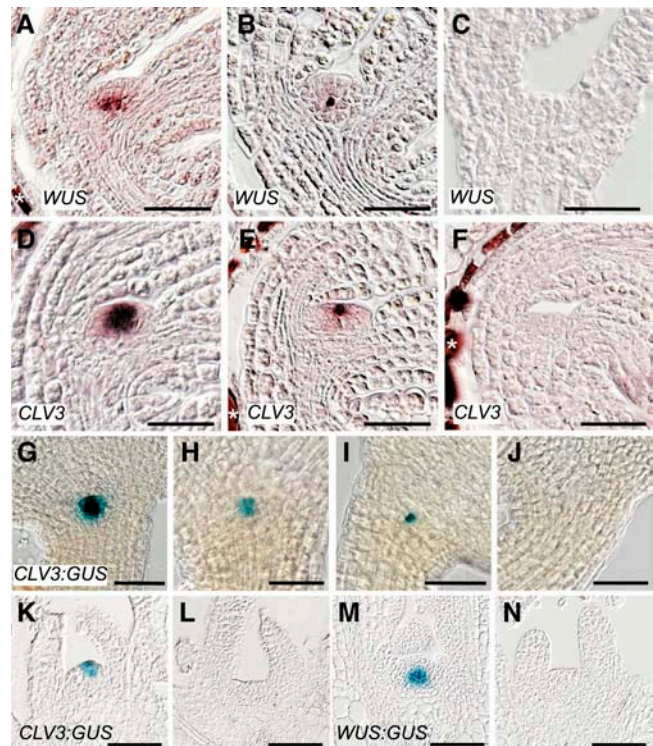

Figure 5. The *l28* Mutation Affects Gene Expression in the Stem Cell Niche.

(A) to **(F)** In situ hybridization in bent-cotyledon-stage embryos in the progeny of an *l28/+* plant. Signal is detected as reddish color. Background staining independent of the in situ procedure is marked (asterisk). *WUS* expression is shown in **(A)** to **(C)**: wild-type-like expression **(A)** and reduced **(B)** or absent **(C)** expression. *CLV3* expression is shown in **(D)** to **(F)**: wild-type-like expression **(D)** and reduced **(E)** or absent **(F)** expression.
(G) to **(J)** GUS-stained embryos from a *CLV3:GUS l28/+* plant. Wild-type-like expression **(G)** and embryos with reduced (**[H]** and **[I]**) or no **(J)** *CLV3* expression.
(K) and **(L)** *CLV3:GUS* expression in 12-d-old seedlings.
(K) *l28* mutant with convex apex and wild-type-like expression.
(L) Mutant in which the apex has lost its dome-like structure and shows no expression.
(M) and **(N)** *WUS:GUS* expression in 12-d-old mutants.
(M) *l28* mutant with wild-type meristem and wild-type-like expression.
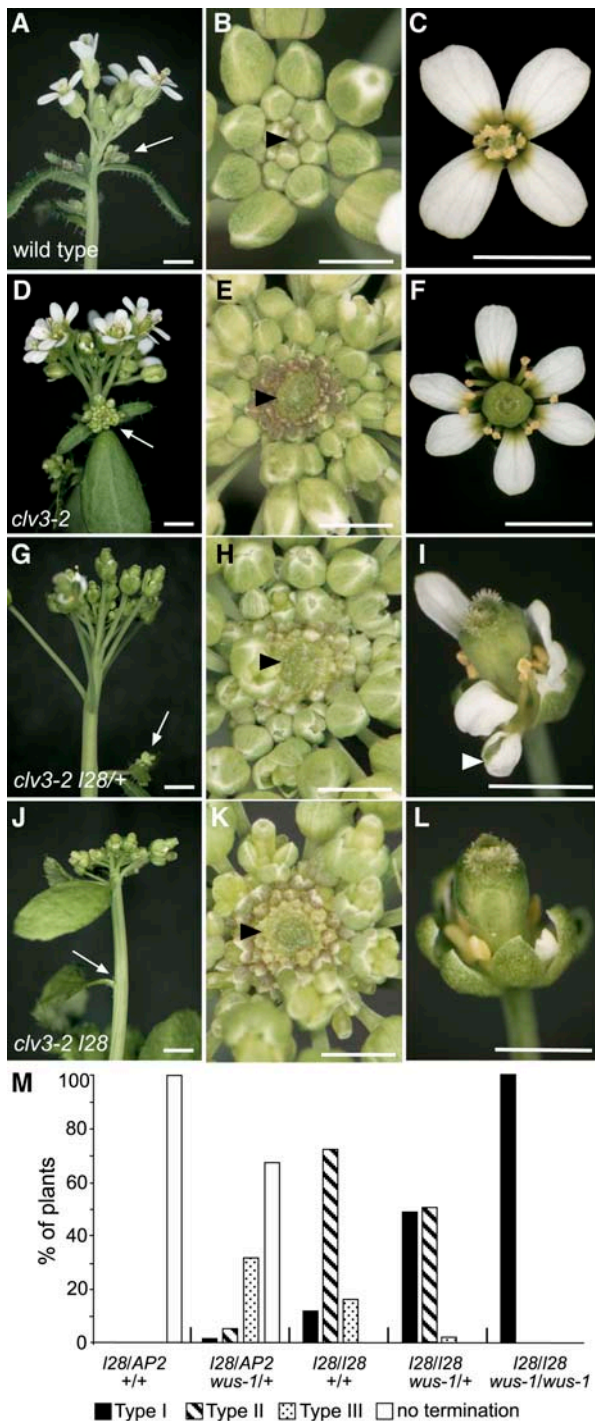**(N)** Mutant seedling with a terminated apex and no expression.
Bars = 50 μm.

of *l28* homozygous seedlings but did not rescue postembryonic axillary meristem formation.

All *clv3-2 l28/+* plants formed an enlarged primary shoot meristem like *clv* single mutants (Figures 6G to 6I). However, similar to *l28/+* heterozygotes alone, these plants rarely formed side meristems in the axils of most rosette leaves (Table 2).

The majority of double mutants between *l28* and the intermediate *clv3-1* allele (Clark et al., 1995) were indistinguishable from *l28 clv3-2* double mutants. However, in contrast with the latter, a minority (2.2%, *n* = 179) of the progeny of *clv3-1 l28/+* plants displayed premature shoot meristem termination, indicating that this allele rescued the *l28* phenotype less efficiently than the strong *clv3-2* allele.

Taken together, these results indicate that strong *clv3* and *clv1* mutations rescue primary shoot meristem formation in *l28*. By contrast, the effects of *l28* on postembryonically formed axillary shoot meristem formation were not rescued, indicating that *AP2* has additional affects on leaf axil development prior to the establishment of the *WUS-CLV3* feedback loop.

AP2 and *WUS* Act in the Same Process in Stem Cell Maintenance

To study the functional relationship between *WUS* and *AP2* in stem cell specification, we analyzed genetic combinations of the putative null allele *wus-1* and *l28* in a segregating progeny of a *wus-1/+ l28/+* transheterozygous plant. All genotypes were determined by dCAPS-PCR (see Methods). Five-day-old *wus-1* seedlings (Laux et al., 1996) are indistinguishable from the Type I *l28* seedlings with regard to the lack of a shoot meristem, absence of *CLV3* expression, and the structure of a flat apex of differentiated cells. However, they differ in leaf formation: *wus-1* seedlings initiate the first two leaves similar to the wild type, albeit delayed, in agreement with the view that the stem cell niche is not required for the formation of the first organs but only for sustained organ formation (Laux et al., 1996). By contrast, Type I *l28* apices only form adventitious leaves in the axils of the cotyledons at ∼14 d after germination (see Figure 1C). This suggests that both mutations disrupt the stem cell niche in the center of the apex in a similar way, whereas only *l28* additionally affects initial leaf formation, consistent with the broader expression domain of *AP2* in comparison with *WUS*.

Gradual reduction of *WUS* activity in a homozygous *l28* background resulted in an increasing severity of shoot meristem termination, with frequencies of the earliest termination (Type I) being 12% in *l28* single mutants, 50% in *l28 wus-1/+*, and 100% in *l28 wus-1* double mutant seedlings (Figure 6M). This indicates

Figure 6. *l28* Double Mutant Analysis.

(A) to **(C)** Inflorescences and flower of a wild-type plant.
(D) to **(F)** Plants that are homozygous for *clv3-2* and wild-type for *AP2* display supernumerary flowers **(D)**, an enlarged shoot meristem **(E)**, and supernumerary floral organs **(F)**.
(G) to **(I)** *clv3-2* mutants heterozygous for *l28* resemble *clv3* plants but additionally have flowers with chimeric floral organs (**[I]**; arrowhead).
(J) to **(L)** The primary shoot meristem of *clv3-2 l28* double mutants does not terminate as in *l28* but enlarges as in the *clv3* single mutant. No side

branches are formed in the axils of the cauline leaves **(J)**. The flowers lack second whorl organs or have sepaloid organs in the second whorl **(L)**.
(M) Dosage-dependent effect of *wus-1* on the stage of meristem termination in heterozygous and homozygous *l28* mutants: Type I, termination before any true leaves have formed; Type II, termination after some true leaves have formed; Type III, termination after a short stem has formed.
Arrows in **(A)**, **(D)**, **(G)**, and **(J)** indicate leaf axils. Black arrowheads in **(B)**, **(E)**, **(H)**, and **(K)** indicate the shoot meristem. Bars = 3.0 mm.

that transient shoot meristem activity in homozygous *l28* mutants before termination occurs is dependent on *WUS* activity in a dosage-dependent manner.

Approximately one-third of *l28/+ wus-1/+* plants also resulted in premature termination of the primary shoot meristem after formation of a few leaves, which was not observed in plants heterozygous for either mutation alone (Figure 6M). Subsequently, these plants were able to form flowering adventitious shoots indistinguishable from inflorescences of *l28/+* heterozygotes alone. The remaining two-thirds of the *l28/+ wus-1/+* plants did not terminate the shoot meristem prematurely but developed similar to *l28/+* heterozygotes alone.

Taken together, *l28 wus-1* genetic combinations indicate that *l28* and *WUS* affect the same process of shoot meristem maintenance. We did not detect any interaction in combinations of *wus-1* and the loss-of-function alleles *ap2-1* and *ap2-2* (see Supplemental Table 3 online), indicating that plants with reduced *WUS* activity are sensitive to *l28* but not to recessive alleles.

Different Domains of the Shoot Meristem Affect the Stem Cell Niche

In the floral meristem, translation of *AP2* mRNA is repressed in the central whorls (Chen, 2004). To address whether *AP2* is functional in the central cells of the primary shoot meristem and in which cells of the shoot meristem *l28* affects stem cell maintenance, we analyzed the effects of *l28* expression in different domains of the shoot apex. All lines analyzed carried one copy of a transgene expressing the synthetic transcription factor LhG4 from different promoters and one copy of a *pOp:l28* transgene that is activated by LhG4 (Moore et al., 1998).

Expression of *l28* in the stem cell region (*CLV3≫l28*, the double arrowheads denote a two-component expression construct; see Methods) resulted in premature shoot meristem termination in a flat apex similar to homozygous *l28* mutants (Types II and III; Figure 7, Table 3), consistent with *l28* mRNA being translated in the central cells of the shoot meristem. Expression of *l28* in the inner cells of the shoot meristem that express the receptor for *CLV3* (*CLV1≫l28*) and in the L1 cells of the shoot apex (*ATML1≫l28*) gave similar results (Figure 7, Table 3). Occasionally seedlings formed a terminal flower (Figure 7E) or gave rise to an inflorescence stem with several flowers before the inflorescence meristem terminated. The flowers on these inflorescences resembled those of *l28/+* plants (data not shown). Notably, expression of *l28* in the stem cell region from the *CLV3* promoter resulted on average in earlier termination of the primary shoot meristem than expression from any other promoter (Table 3). Nevertheless, even in this case we did not observe the most severe Type I phenotype that we found in homozygous *l28* mutants.

In contrast with the activator lines mentioned above, expression of *l28* in the OC under the control of the *WUS* promoter did not give a shoot meristem phenotype in plants that were heterozygous for both *WUS:LhG4* and *pOp:l28* (Table 3). We therefore analyzed F2 plants and found that in one-sixteenth of the progeny the inflorescence meristem prematurely terminated in a flat apex after the formation of a few flowers (Figures 7H and 7I). This segregation ratio suggests that only the plants that were
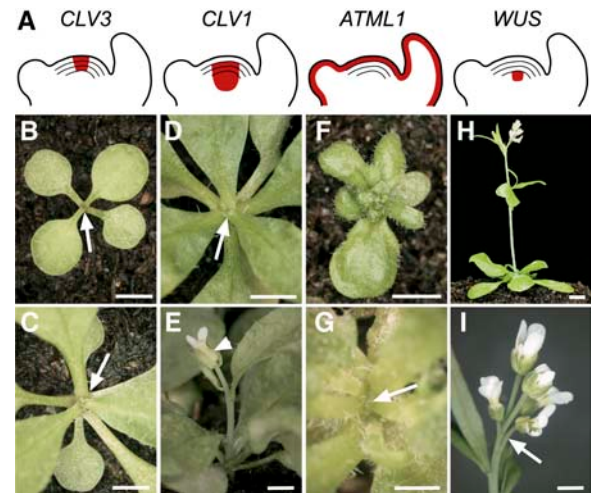

Figure 7. Ectopic Expression of *l28* in Subdomains of the Shoot Apex.

(A) Expression domains of used transgenes.

(B) and **(C)** Expression of *l28* under the *CLV3* promoter results in premature termination of the shoot meristem after a few leaves have formed (Type II).

(D) and **(E)** When driven by the *CLV1* promoter, the shoot meristem terminates after formation of several leaves **(D)** or production of a terminal flower (arrowhead in **[E]**).

(F) and **(G)** *ATML1≫l28* plants terminate the primary shoot meristem and display curled leaves **(F)**.

(H) and **(I)** In presumably doubly homozygous *WUS≫l28* F2 plants, the inflorescence meristem is terminated after the formation of a few flowers. **(I)** is a close-up of the plant shown in **(H)**.

Arrows indicate prematurely terminated shoot apex. Bars = 5.0 mm, except **(G)**, which is 1.0 mm.

homozygous for both constructs prematurely terminated shoot meristem development. The relatively weak effects correlate with weaker expression levels conferred by the *WUS:LhG4* activator line in comparison with the other activator lines used in this experiment (see Supplemental Figure 2 online).

In control experiments, the effects of *l28* expression in all meristem regions were alleviated by coexpression of wild-type *AP2* in the respective subdomains, suggesting competitive interference between both activities. Transgenic plants with a mutant version of *l28* with all three reading frames disrupted were aphenotypic, demonstrating that the effects were not caused by cosuppression (data not shown).

In conclusion, *l28* and *AP2* mRNA were active in the central region of the shoot meristem, and the effects of *l28* expression in different subdomains of the shoot meristem suggest that *AP2* or a factor(s) regulated by it affect the stem cell niche via non-cell-autonomous mechanisms.

DISCUSSION

Continuous organ formation from the shoot meristem during the postembryonic life of higher plants depends on the maintenance of undifferentiated stem cells in specific microenvironments, named stem cell niches (Weigel and Jürgens, 2002; Laux, 2003).

Table 3. *l28* Expression in Subdomains of the Shoot Meristem

Transgenes	Type I *n*	Type II								Type III Late Stop	Terminal Flower	No Termination
		1	2	3	4	5	6	7	8			
CLV3≫l28	31		23	42	13	19	3				0	0	0
CLV1≫l28	55					2	2	16	9	42	27	2
ATML1≫l28	17				12	18	29	23	6	0	6	6
WUS≫l28	35									0	0	100

Developmental time point of plants showing a meristem arrest (in percent) in plants ectopically expressing *l28*. F1 plants from a cross between the activator line and the *pOp:l28* line were analyzed.

Here, we identify a novel regulatory pathway in shoot meristem stem cell maintenance defined by *AP2* function.

Stem Cell Maintenance Is Regulated by *AP2*

AP2 was originally identified as a floral homeotic gene encoding A-function of the ABC model of organ identity specification (Bowman et al., 1989, 1991). Recently, it has also been implicated in floral transition and control of seed size (Okamuro et al., 1997b; Jofuku et al., 2005; Ohto et al., 2005). Here, we show that *l28* and *l28/+* plants have a number of phenotypes in common with *ap2* loss-of-function alleles, such as early flowering, mispecified floral organs, and a reduction in shoot meristem size. By contrast, premature termination of the shoot meristem and differentiation of the stem cells is only observed in the *l28* allele. Wild-type *AP2* is expressed in the shoot meristem at all stages, consistent with an as yet unknown function there. Our gene dosage experiments indicate that wild-type AP2 activity competes with l28 activity. A plausible interpretation of these data is that *l28* acts as a dominant-negative allele of *AP2* and thus identifies a novel pathway in shoot meristem development. Definite proof of this model has to await the identification of potential redundant factors that appear to mask *AP2* function in the shoot meristem.

The *l28* mutation changes a highly conserved negatively charged Glu residue into a positively charged Lys residue within the first of the two putative DNA binding domains of AP2. A possible model is therefore that the mutant protein competes with wild-type AP2 and redundant factor(s) for interactors in a protein complex but compromises its binding to DNA targets. Alternatively, the mutant protein may still be able to bind to AP2 target genes but fail to interact with cofactors required for transcriptional regulation. Stochastic variations in the balance between mutant and redundant wild-type proteins might account for the differences when meristem termination occurs in individual homozygous *l28* mutants.

AP2 Functions to Maintain the Stem Cell Niche in the *Arabidopsis* Shoot Meristem

How does *AP2* regulate stem cells? Our results indicate that termination of the primary shoot meristem in *l28* mutants is not caused by a shortage of cells but by the failure to specify the undifferentiated state of stem cells. *WUS* and *CLV3* expression is disrupted in *l28* mutants from early embryo stages on, and *l28* primary shoot meristems terminate in flat apices where the cells have undergone partial differentiation indistinguishable from *wus* seedlings. Both *CLV3* and *AG* function as repressors of *WUS* and stem cell maintenance. However, unlike in floral patterning, *AP2* functions in shoot meristem stem cell maintenance by a mechanism independent of *AG*. By contrast, termination of the shoot meristem by *l28* requires an active *CLV3* gene. Therefore, a plausible interpretation of these data is that *AP2* or a factor(s) regulated by it affect stem cell maintenance in the primary shoot meristem by negatively regulating the *CLV* signaling pathway (Figure 8).

According to this model, dominant inhibition of *AP2* activity and redundant factors in *l28* mutants would result in upregulated *CLV* signaling, which causes enhanced repression of *WUS* and in turn downregulation of *CLV3* expression. Consequently, *l28* would have no effect on the stem cell niche in a *clv1* or *clv3* mutant background. Since we never observed an increase of *CLV3* expression in sectioned apices of *l28* mutants, as expected if *AP2* negatively regulated *CLV3* promoter activity, *AP2* appears to affect CLV signaling downstream of *CLV3* transcription.

Alternatively, *AP2* and *CLV3* could act antagonistically but independently (Figure 8). However, such a mechanism would
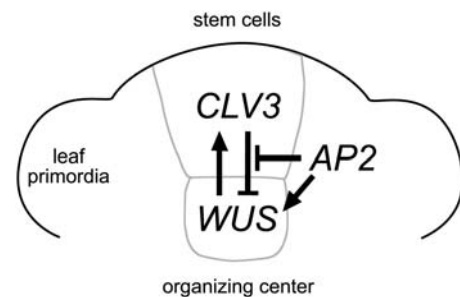

Figure 8. Model for AP2 Action in the Stem Cell Niche of the Primary Shoot Meristem.

AP2 could promote stem cell maintenance either by negatively regulating CLV signaling (blunt-ended bars) or by independently antagonizing its effects (arrows).

require the absence of *CLV3* signaling to have a far greater effect on *WUS* expression than the inhibition of *AP2* in *clv3 l28* double mutants to explain not only the rescue of primary shoot meristem formation but also meristem enlargement similar to *clv3* single mutants.

Our results suggest that *AP2* can affect the stem cell niche from different regions of the shoot meristem. It is thus possible that this pathway serves to integrate information from surrounding shoot meristem regions to tune the activity of the stem cell niche. The future identification of target genes of *AP2* will facilitate exposure of the precise molecular mechanism underlying its function in stem cell control.

Redundancy of *AP2* Function in Stem Cell Regulation

The factors that mask AP2 function in stem cell maintenance in recessive hypomorphic alleles are yet to be identified. Our ongoing genetic studies suggest that the two closest homologs, *TOE3* and *TOE1* (Aukerman and Sakai, 2003), do not act redundantly to AP2 in stem cell maintenance (T. Würschum and T. Laux, unpublished data). Some members of the AP2/ERF family have been associated with the regulation of cell differentiation (Boutilier et al., 2002; Kirch et al., 2003). Among these, the *DORNRÖSCHEN* gene affects shoot meristem development but appears to have opposite and more pleiotropic effects compared with *AP2* (Kirch et al., 2003). Intriguingly, *PLETHORA1* and *PLETHORA2* genes have been found to play an important role in regulating the stem cell niche of the *Arabidopsis* root meristem and to act redundantly (Aida et al., 2004). Together with the evidence presented here, these findings suggest that some members of the *AP2* subfamily represent conserved units in the regulation of stem cell niches.

METHODS

Plant Growth, Mutant Lines, and Mapping

All plants were in the Landsberg *erecta* (L*er*) ecotype, which was also used as the wild-type control. Growth conditions have been described previously (Laux et al., 1996). The *l28* mutant was identified in the M2 of ethyl methanesulfonate–mutagenized wild-type plants and subsequently backcrossed three times to wild-type L*er* before further analysis. *clv1-4*, *clv1-10*, *clv3-1*, and *clv3-2* (Clark et al., 1993, 1995), *wus-1* (Laux et al., 1996), *stm-5* (Endrizzi et al., 1996), and *ap2-1* and *ap2-2* mutants have been described (Bowman et al., 1989, 1991).

The *l28* mutation was mapped using plants with a wild-type-like phenotype in the F2 from a cross of an *l28*/+ plant with the Columbia ecotype. The initial mapping was done using CAPS markers from The Arabidopsis Information Resource (http://www.arabidopsis.org). The dCAPS markers used for fine mapping of the *l28* mutation were generated based on the information available by CEREON (Jander et al., 2002). Details about primer sequences and polymorphisms are available upon request.

Histology, GUS Staining, and in Situ Hybridization

Preparation of histological sections from LR-White embedded material (Laux et al., 1996), GUS staining (Schoof et al., 2000), and in situ hybridization were previously described (Mayer et al., 1998).

For the *AP2* riboprobe, the C-terminal part of *AP2* lacking both AP2 domains was amplified from a L*er* cDNA library using primers AP2 FOR (5′-GCCGAGTCATCAGGGAATCCTAC-3′) and AP2 Op REV (5′-GCAAG-ATCTAAGCCGATGATTAATGAAATGACC-3′) and subcloned into pGEM-T (Promega) to yield TW44. For the antisense probe, TW44 was linearized with *Sal*I and transcribed with T7 RNA polymerase (Promega) using a digoxigenin-labeling kit (Roche Diagnostics); for the sense probe, TW44 was linearized with *Nco*I and transcribed with SP6 RNA polymerase (Promega).

Construction of Transgenes and Plant Transformation

To generate the *AP2:AP2* and *AP2:l28* constructs, the *AP2* and *l28* genomic regions were amplified using primers AP2 FOR (5′-GTCGACGGA-GAATCATACCAGAGGATTGAAGTTTG-3′) and AP2 REV (5′-CCAATGT-TAGATCTAGGAAACCATGTGTATACC-3′) and subcloned into pGEM-T (Promega) to yield TW28 and TW29. Both TW28 and TW29 were sequenced to exclude amplification errors. As promoter, 5 kb of upstream sequence was amplified using primers AP2 Prom FOR (5′-GTCGACTCCTTACTA-TATTTGTACATTAACGTGTTCC-3′) and AP2 Prom REV (5′-GTCGACC-CTCAAGGTTAGAAGCTAAATAAGAGAAACC-3′) and subcloned into pGEM-T to yield TW26. The promoter was excised from TW26 by digestion with *Sal*I and cloned into TW28 and TW29 each digested with *Sal*I/CIAP to yield TW35 and TW37. TW35 and TW37 were digested with *Nco*I/T4 and *Sac*I, and the resulting *AP2:AP2* and *AP2:l28* inserts were cloned into pBarM (Lenhard et al., 2002) to yield TW51 and TW54.

For the *AP2:GUS* reporter, the same 5 kb were amplified using primers that introduced *Pst*I restriction sites AP2 Pd FOR (5′-CCTGAGTAAT-CATTTGTCCTGCAGCCATATC-3′) and AP2 Pd REV (5′-GTTCAATCTG-CAGTCCTCTGGTATGATTCTC-3′). The resulting PCR product was digested with *Pst*I and cloned into the *Pst*I site of mL255 to yield TW49.

For all misexpression studies, we used the *pOpL* two-component system where the synthetic transcription factor LhG4 is expressed under the control of the promoter of interest (Moore et al., 1998). The gene to be misexpressed is under the control of the *pOp* promoter, which is specifically activated by LhG. In every case, transgenic lines carrying either the activator or the target construct alone did not show a phenotype. The phenotypes that are described were only observed when both the activator and the target were brought together in one plant by crossing. Both the *pOp:l28* and the *pOp:AP2* lines used in these experiments carried a linked *pOp:GUS* to monitor the expression of the target construct.

For the *pOp:AP2* and *pOp:l28* constructs, TW28 and TW29 were digested with *Sal*I and *Bgl*II, and the resulting *AP2* and *l28* inserts were cloned into mL204 (Lenhard and Laux, 2003) digested with *Xho*I and *Bam*HI to yield TW40 and TW42. For the negative control constructs *pOp:nsAP2* and *pOp:nsl28*, an analogous fusion was created using cDNAs that contained stop codons in all three reading frames.

The *WUS:LhG4*, *CLV1:LhG4*, *CLV3:LhG4*, and *ATML1:LhG4* driver lines have been described (Schoof et al., 2000; Lenhard et al., 2001; Lenhard and Laux, 2003).

All plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90; Koncz and Schell, 1986) by electroporation and transformed into L*er* wild-type plants by the floral dip method (Clough and Bent, 1998).

PCR-Based Genotyping

Plants were genotyped for the *l28* allele by dCAPS (Neff et al., 1998) using primers *l28* FOR (5′-GGGCTCACTGGTATGGTGTTAAGT-3′) and *l28* REV (5′-TAAAGATATGAGATTATTACCAAATATGGAATT-3′) at an annealing temperature of 53°C. Digestion of the PCR products with *Eco*RI produces two bands of 189 and 33 bp from the product of the wild-type allele, while the product from the *l28* allele is 222 bp and is not digested. For the *wus-1* allele, the primers WUS1 FOR (5′-TAGTATGGTCTGGATTCTGGAATC-3′) and WUS1 REV (5′-TATTTGTATTAATGAATTATAGTTTGATACGTA-3′)

were used. Digestion with *Bsa*BI gives a 193-bp wild-type fragment and 165- and 28-bp fragments for the *wus-1* allele.

Shoot Meristem Size Measurements

Confocal analysis of embryos (Running et al., 1995), scanning electron microscopy, and inflorescence meristem size measurements were done as previously described (Lenhard and Laux, 2003).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers are as follows: *AP2* (At4g36920), *TOE1* (At2g28550), and *TOE3* (At5g67180).

Supplemental Data

The following materials are available in the online version of this article.

   Supplemental Table 1. Transformation of *AP2* and *I28* Genomic DNA.

   Supplemental Table 2. *I28 ag-1* Double Mutant Analysis.

   Supplemental Table 3. *ap2 wus-1* Double Mutant Analysis.

   Supplemental Figure 1. Comparison of Hypocotyl and Root Size between *I28* and the Wild Type.

   Supplemental Figure 2. Expression Strength of the Activator Lines.

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