Dynamic and Compensatory Responses of Arabidopsis Shoot and Floral Meristems to CLV3 Signaling

Ralf Müller, Lorenzo Borghi, Dorota Kwiatkowska, Patrick Laufs, and Rüdiger Simon

In Arabidopsis thaliana, the stem cell population of the shoot system is controlled by regulatory circuitry involving the WUSCHEL (WUS) and CLAVATA (CLV1-3) genes. WUS signals from the organizing center (OC) to promote stem cell fate at the meristem apex. Stem cells express the secreted peptide CLV3 that activates a signal transduction cascade to restrict WUS expression, thus providing a feedback mechanism. Stem cell homeostasis is proposed to be achieved by balancing these signals. We tested the dynamics of CLV3 signaling using an inducible gene expression system. We show here that increasing the CLV3 signal can very rapidly repress WUS expression during development, which in turn causes a fast reduction of CLV3 expression. We demonstrate that increased CLV3 signaling restricts meristem growth and promotes allocation of peripheral meristem cells into organ primordia. In addition, we extend the current model for stem cell control by showing that meristem homeostasis tolerates variation in CLV3 levels over a 10-fold range and that high-level CLV3 signaling can be partially compensated with time, indicating that the level of CLV3 expression communicates only limited information on stem cell number to the underlying OC cells.

INTRODUCTION

Plant meristems provide the protective niche that allows maintenance and proliferation of undifferentiated stem cells. Intercellular communication between the functional domains of the meristem allows them to maintain their size and shape during development. Organs are formed at the meristem flanks in the peripheral zone (PZ), and stem cell divisions in the central zone (CZ) have to provide new cells to support continuous organ formation. In Arabidopsis thaliana, stem cell fate in the CZ of shoot and floral meristems is promoted by the cells of the underlying organizing center (OC) (Clark, 2001a; Baurle and Laux, 2003; Carles and Fletcher, 2003) that express the homeodomain transcription factor WUSCHEL (WUS) (Mayer et al., 1998). In wus mutants, stem cells are no longer maintained, and organ formation arrests prematurely (Laux et al., 1996). WUS expression is repressed by the CLAVATA (CLV) signal transduction pathway (Clark, 2001b; Carles and Fletcher, 2003). Stem cells in the CZ express the secreted peptide CLV3 that is thought to bind to and thereby activate the CLV1 leucine-rich receptor kinase in cells underneath (Fletcher et al., 1999; Brand et al., 2000). Mutations in CLV1 or CLV3 cause stem cell accumulation in the CZ due to unrestricted WUS expression, resulting in a larger surrounding PZ and the formation of more organs.

A model of how stem cell number in the CZ is controlled by feedback regulation between CLV3 and WUS has been proposed and predicts that if stem cell number decreases, less CLV3 is produced and WUS is released from repression by the CLV pathway. On the other hand, if stem cell number increases, CLV3 signaling intensifies and downregulates WUS expression (Brand et al., 2000; Schoof et al., 2000). A range of transgenic plants expressing CLV3 or WUS from different promoters has been generated, and the phenotypes observed support the current model. However, the dynamics of the regulatory system are only poorly understood. One recent study showed that inactivation of CLV3 during development using an RNA interference (RNAi) approach allows for unrestricted WUS expression and activation of ectopic CLV3 expression in PZ cells (Reddy and Meyerowitz, 2005).

Here, we have analyzed the dynamics of altered CLV3 signaling and the consequences for WUS expression and meristem development. CLV3 belongs to the CLE family of small signaling molecules that can, in part, functionally substitute for each other, provided that they are expressed at sufficient levels in a given cell (Casamitjana-Martinez et al., 2003; Hobe et al., 2003). To avoid artificial crosstalk between signaling pathways due to high-level misexpression, we modulated CLV3 expression levels within its normal expression domain. We show here that WUS expression decreases very rapidly when CLV3 expression increases. However, we found no direct proportionality between the levels of the stem cell CLV3 signal and WUS repression. We extend the existing model for stem cell regulation and propose that WUS
activity in the OC is controlled by a secondary mechanism that can compensate for fluctuations in CLV3 expression with time. Furthermore, we show that WUS activity or an active stem cell population is required to restrict primordia initiation to the meristem’s periphery.

RESULTS

Inducible Expression of CLV3 in the Stem Cell Domain

We used the ethanol-inducible gene expression system to activate CLV3 expression during development (Deveaux et al., 2003). Briefly, the ALCR transcription factor was expressed under control of the CLV3 regulatory sequences (named CLV3:ALCR). Target genes to be expressed in an inducible manner are cloned behind the alcA promoter sequences and introduced into the same plant. An alcA:β-glucuronidase (GUS) transgene was used as a reporter for successful activation of gene expression via the ALC system. The alcA:CLV3 transgene allowed inducible expression of CLV3 within the stem cell domain. Transgenic Arabidopsis plants that carried the CLV3:ALCR, alcA:GUS, and alcA:CLV3 transgenes were obtained (iCLV3). Histochemical staining for GUS reporter gene activity and RNA in situ hybridization using GUS probes confirmed that the reporter gene was inducible but not active prior to ethanol induction (Figures 1C and 6F) (Deveaux et al., 2003). Inductions using ethanol vapor were performed between 5 and 7 weeks after germination at or after the onset of bolting. Continuous inductions caused termination of shoot meristem development in both wild-type and clv3-2 mutant backgrounds, as expected from high expression of CLV3 (Figure 1B). Control plants lacking the alcA:CLV3 transgene, as well as wild-type Landsberg erecta plants, were unaffected by the inductive treatments (Figure 1A).

To study the consequences of transient changes in CLV3 signaling on meristem development, we first had to identify an appropriate length for the inductive treatment. Therefore, the percentage of plants showing shoot meristem termination after different inductive periods was determined. No termination was observed in plants treated for 2 h. Inductions for 6 to 24 h resulted in 5 to 7% meristem termination; treatments for 3 d caused shoot meristem arrest in 78% of all plants, and inductions for 6 or 12 d caused termination in all plants analyzed (total n = 458). To allow for shoot apical meristem (SAM) recovery in the majority of plants, we performed single pulse treatments of 6-h duration. Quantitative RT-PCR of CLV3 levels showed that expression was rapidly induced within 3 h and that CLV3 expression levels remained elevated for up to 48 h after induction (HAI) (Figure 2A). Concomitantly, WUS RNA amounts decreased within 3 HAI to a minimum of 40% between 6 and 22 HAI. Close to or even higher than normal WUS RNA levels were reached again within 48 h.

Plants were allowed to grow to maturity after the single pulse to study morphology and flower organ number. On 15% of all induced plants, two to three sterile flowers that lacked one or both carpels developed somewhere along the main inflorescences axis, followed by normal fertile flowers (Figures 1D and 3A). Thus, only a minority of all flower meristems responded to a rapid, short-term pulse in CLV3 signaling with a reduction in stem cell activity.

We also noted that flower arrangement along the stem axis appeared altered above the position of sterile flowers (Figures 1D, 1E, and 3A). In 59% of treated plants, three to six flowers were arranged in small clusters (n = 86), which could be due to a transient alteration in meristem size or a transient failure in internode elongation. Interestingly, wus mutant plants initiate so-called aerial rosettes, which are clusters of leaves arranged in a dense spiral (with short internodes) around an inflorescence stem (Mayer et al., 1998). However, dissecting the SAM together with the youngest flower primordia from wild-type Landsberg erecta plants (n = 26) resulted in the formation of similar flower clusters, indicating that an active SAM is required for internode elongation (Figure 1F).

Our pulsed induction experiments showed that a transient increase in CLV3 signaling is not generally sufficient for immediate termination of shoot meristem development. We found that induction for >3 d is required to arrest SAM growth in all plants.

WUS Is Rapidly but Transiently Repressed after Induction of CLV3

Only some of the floral meristems responded to increased CLV3 expression with a reduction in meristem activity, which could be due to the limited length of the inductive treatment. We therefore performed continuous inductions with ethanol for 6 h per day for 12 d. Within 3 h after commencing ethanol induction, the CLV3 RNA level increased 70-fold and increased further to >400 times the uninduced level within 72 HAI (Figure 2B). Quantification of WUS RNA levels in shoot tips, comprising inflorescence and floral meristems, showed an overall reduction to 35% of untreated levels within 24 HAI (Figure 2B). However, WUS RNA remained always detectable. We observed a significant increase in WUS RNA amounts from 72 HAI onwards, which could at least in part be due to the expression of WUS in stamens and ovolves that now start to develop. There, WUS expression is not subject to regulation via the CLV pathway (Gross-Hardt et al., 2002; Wellmer et al., 2004). Since CLV3 expression depends on WUS activity, we expected that downregulation of WUS expression in shoot and floral meristems would be paralleled by reduced CLV3 expression. To distinguish between CLV3 expression from the transgene and the endogenous gene copy, we performed quantitative RT-PCR analysis using gene-specific amplification primers derived from untranslated region sequences (Figure 2B). Expression of the inducible CLV3 (from the CLV3:ALCR; ALCA:CLV3 transgene), which is controlled from a CLV3 promoter and therefore ultimately dependent on WUS activity, is maintained for at least 168 HAI due to the stability of the ALCR mRNA (data not shown). Expression from the endogenous CLV3 gene was significantly reduced within 3 HAI and close to background by 24 HAI (Figure 2B), in line with the expectation that WUS is required to promote CLV3 expression. Surprisingly, CLV3 expression levels increased again by 72 and 168 HAI, which could suggest that WUS was also reactivated in meristems (Figure 2B).

Phenotypic Consequences of Long-Term CLV3 Induction

At the macroscopic level, differences from untreated plants became apparent 9 d after induction (DAI). All plants that were
induced for six or more consecutive days arrested shoot growth and flower formation (>250 plants analyzed) (Figures 1G to 1J). Internode elongation between the last three to six flowers was strongly reduced, resulting in a pseudo-whorled arrangement of flowers around the shoot tip, thus resembling pulse-induced plants. In some cases, a small filamentous organ grew at the arresting shoot apex (Figure 1). We analyzed all flowers that were formed on terminated iCLV3 plants (n = 32) after long-term, continuous ethanol induction (Figure 3B). Although floral induction was synchronized by shifting plants from short days to long days at 43 d after germination (DAG), individual plants still differed in their developmental stage and in their total number of flowers, ranging from 3 to 21. In 26 of 32 plants (81%), we found one to six flowers carrying three to four stamens and only one carpel, indicating a premature floral meristem arrest. The distribution of these flowers along the inflorescence axis depends on the developmental stage at which the induction of CLV3 expression started (Figure 3B). Defective flowers were
formed only for a short period, after which, on average seven normal flowers were grown higher up on the inflorescence. Thus, while the shoot meristem of all plants responded to strong CLV3 signaling with stem cell loss, only a limited number of floral meristems reacted in a similar manner. A similar restricted response was observed when CLV3 was inducibly and ubiquitously expressed using the cauliflower mosaic virus 35S promoter (J. Lohmann, personal communication). This could indicate that CLV3 can act only during a narrow developmental window. Such a window must lie between stages 1 and 6 of flower development, when WUS is normally expressed. However, on average seven normal flowers are found higher up on the inflorescence of our iCLV3 plants, which were formed after the defective flowers. Notably, the youngest flowers passed through all developmental stages during the ethanol treatment. We therefore conclude that the response to CLV3 signaling cannot depend on a sensitive stage of flower development. We also considered the possibility that the iCLV3 transgene is silenced during plant development. However, plants that had been induced once with a 6-h single ethanol pulse still responded to a second, inductive pulse provided 10 d later (data not shown), confirming that the transgene could still be activated.

Increased CLV3 Signaling Reduces SAM Growth and Recruits More SAM Cells to Primordia

In order to visualize the dynamic cellular response to increased CLV3 signaling, we prepared replicas (developmental sequences) of the meristem surface before and at regular intervals after the inductive treatment, which were then analyzed by scanning electron microscopy (Dumais and Kwiatkowska, 2002). To control for tissue damage induced by the imprinting technology, we repeatedly took replicas from individual untreated apices, without

**Figure 2.** Quantification of CLV3 and WUS Expression after iCLV3 Induction.

Total RNA was isolated from shoot tips of induced plants at the indicated time points, and CLV3 or WUS transcripts were quantified by quantitative RT-PCR. Two independent RNA preparations were analyzed for each time point. Median values were calculated from triplicate real-time PCR analysis, and standard errors are shown. Levels of CLV3 and WUS RNAs before induction (time point 0) were set to 1. The y axis represents X-fold induction in a logarithmic scale. Means are of three experimental repetitions, and bars indicate the standard error.

(A) Changes in CLV3 and WUS expression after a single 6-h ethanol pulse.

(B) Changes in CLV3 and WUS expression after continuous inductions. Oligonucleotide primers were used that allowed iCLV3 transgene (induced CLV3) and CLV3 gene (endogenous CLV3) expression to be distinguished.

**Figure 3.** Effects of CLV3 Induction on Plant Development.

(A) Pulsed induction. iCLV3 transgenic plants were induced for 6 h, and the morphology of the first 15 flowers was analyzed for individual induced plants and plotted according to their position on the inflorescence axis. Gray, normal flowers with two carpels; black, 0 or 1 carpel; white, aberrant positioning of flowers (i.e., more than three flowers are formed within 3 mm of stem axis). Only plants affected by the induction were plotted (51 of 86).

(B) Continuous inductions. Columns represent individual iCLV3 plants analyzed after shoot meristem termination. Flowers along the inflorescence axis were scored for carpel number. Gray, normal flower with two carpels; black, flower with 0 or 1 carpel; white, filamentous organ.

Individuals were ordered along the x axis by total number of flowers produced and by the position of the first affected flowers along the inflorescence axis.
noticing any effect on growth or development (Figures 4A1 and 4A2; see Supplemental Figure 1 online). Furthermore, developmental sequences of individual apices were very similar. Overall growth was quantified as the relative area increase of an individual cell or SAM surface (percentage of increase per hour), and numbers of cell divisions in a 24-h interval were plotted on the SAM surface (Figures 4A and 4B). In general, overall growth is higher in primordia than in the center of the meristem, except in regions where flower primordium boundaries are formed (Figure 4, blue in the center and red in primordia).

Within 1 to 3 d after the beginning of induction, overall growth across the CZ and PZ of induced SAMs declined. While division rates in the PZ and primordia were not strongly affected, cell divisions in the meristem center ceased (Figures 4B1 to 4B3). The SAM area started to decrease within this period (Figure 5). Approximately 5 DAI, the arresting SAMs had also changed shape and became flat or saddle shaped (Figure 4B3). The initiation of individual primordia was delayed, with an increase in the plastochron from 10 h in controls to 25 h in induced apices (Table 1). Furthermore, primordia were initiated closer to the SAM center and were significantly larger than those on control apices at the time of initiation (Table 1). Thus, a size decrease of the SAM was paralleled by a size increase of the individual primordia, indicating that increased CLV3 signaling allowed the recruitment of cells from the flanks of the central region for organ initiation.

Figure 4. Analysis of Cell Behavior.

Scanning electron micrographs were taken from meristem replicas every 24 h, and cells were color-coded according to their areal growth rates within this time period (relative size increase, units % h⁻¹). Cell divisions occurring within 24 h are indicated by number of black dots per cell. Asterisks identify the cell at which the geometric center of the SAM surface is located at the beginning of the series. Flower primordia are initiated in a clockwise pattern and are numbered from the youngest primordium (P0) observed in the sequence to the oldest. Their borders as assessed by surface curvatures are outlined in black. Bars = 30 μm.

(A) Sequence of an untreated control meristem. (B) Sequence of an induced meristem. Replicas were taken at days 2 (B1), 3 (B2), and 4 (B3) after the start of induction.
Figure 5. Size Reduction of the SAM Surface Area after CLV3 Induction.
Surface areas (in $\mu$m$^2$) were measured from scanning electron micrographs for four induced apices (dashed lines) at 0 to 6 DAI, and two control meristems (solid lines) were analyzed on consecutive days.

In all shoot meristems analyzed, at least three new flower primordia were initiated after ethanol induction, but before the SAM ceased growth. These flowers developed through all stages during the induction period and were therefore continuously subjected to high CLV3 signaling.

Differential Responses of Floral Meristems to CLV3 Signaling

To characterize the molecular events in more detail, we analyzed the distribution of CLV3 and WUS RNA in tissue sections of induced plants (Figure 6). In the wild type, CLV3 is expressed in a roughly conical domain in shoot and floral meristems, and this general expression pattern remained unaltered by the inductive treatment (Figure 6A). We noted a minor expansion of CLV3 expression into more lateral positions, which can be explained by the stability of ALCR mRNA and inheritance to the flanking stem cell daughters. However, intensity of staining increased dramatically within 6 HAI, as predicted from the RNA quantifications. During the following days, CLV3 remained expressed at high levels in shoot and floral meristems (Figures 6B to 6E). WUS is normally expressed in the OC cells that form a central domain within inflorescence and floral meristems (Figure 6G). In the wild type, WUS is downregulated during stage 6 of flower development, when the gynoecium is formed (Lenhard et al., 2001; Lohmann et al., 2001). In induced plants, WUS expression in the SAM was reduced by 6 HAI and was permanently lost from all SAMs within 24 HAI (Figures 6G to 6I, Table 2). Floral meristems responded differently to increased CLV3 expression: between 6 and 24 HAI, the WUS RNA signal was weak or absent in stage 2 floral meristems and was not detected in stage 3 to 6 meristems (Figures 6H and 6I, Table 2). However, from 48 to 96 HAI, WUS was expressed in 50 and 69% of all stage 2 floral meristems and in 27 and 67% of all stage 3 to 6 meristems, respectively. WUS expression in the SAM had already terminated 3 d earlier, within 24 h after induction had started (Figures 6J to 6K, Table 2). Importantly, WUS expressing flower meristems from all time points after the start of induction still expressed high levels of CLV3 RNA (Figure 6E). The presence of WUS transcripts in floral meristems from 48 HAI onwards could explain that developmental defects were restricted to a limited number of flowers in continuous induction experiments. Thus, increased CLV3 signaling appears to rapidly downregulate WUS expression within 3 h, which also causes a rapid decrease in endogenous CLV3 expression from stem cells. However, downregulation of WUS is at least partially compensated within 2 DAI. This compensatory effect is not limited to floral meristems but was also found in axillary meristems that initiated during the inductive treatment. We conclude that a compensation process, resulting in maintenance or reactivation of WUS expression in the presence of strong CLV3 signaling, acts in all meristems that initiated during the induction period.

CLV3 Expression Can Fluctuate 10-Fold without Altering Meristem Size

The presence of a compensation mechanism that controls WUS expression independently of the CLV signaling pathway would make meristem size regulation at least partially independent of CLV3 expression levels. To test for the effects of different CLV3 expression levels, we made use of a series of CLV3 promoter deletion derivatives that allow expression within the stem cell domain, albeit at reduced or increased levels compared with the wild type (U. Brand, L. Borghi, and R. Simon, unpublished data). In contrast with inducing CLV3 expression rapidly to high levels using the ICLV3 system, we now provide meristems with slightly different amounts of CLV3 continuously from the establishment of the meristem onwards.

We first used a GUS reporter gene to assay promoter activity (Figure 7). The pCLV3 transgene, consisting of −3-kb regulatory sequences controlling expression of a GUS cDNA, gave a GUS RNA expression pattern that corresponded to the RNA distribution of CLV3 in the stem cell domain. All other promoter variants were similarly tested in fusions with the GUS reporter for their dynamic response to CLV3 signaling.

Table 1. Spacing, Timing, and Size of Flower Primordium Formation

<table>
<thead>
<tr>
<th>Spacing</th>
<th>Plastochron Duration (h)</th>
<th>$n_1$</th>
<th>Size ($\mu$m$^2$)</th>
<th>$n_2$</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>4.04 ± 0.11$^{ac}$</td>
<td>10</td>
<td>27</td>
<td>870 ± 141*</td>
</tr>
<tr>
<td>Induced</td>
<td>3.10 ± 0.07*</td>
<td>25</td>
<td>59</td>
<td>1529 ± 109*</td>
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Primordia spacing (distance from SAM center) is given as a number of cells counted from the geometric center of the SAM to the boundary of a flower primordium defined by curvature.

a Flower primordia considered for spacing analysis were the youngest three primordia surrounding the SAM; total number = $n_1$.

b For size measurements, primordia were at the earliest discernible developmental stage; total number = $n_2$.

c Differences between values marked with asterisks are significant at P < 0.05 level of Tukey’s honestly significant difference mean-separation test. Values are means ± SE.
expression patterns, and fluorometric GUS activity assays were performed with at least 20 individual seedlings 10 DAG for each transgenic line to quantify expression levels. We then selected five transgenes that gave transgenic line to quantify expression levels. We then selected performed with at least 20 individual seedlings 10 DAG for each expression patterns, and fluorometric GUS activity assays were incubated overnight; all others gave a strong signal after 6 h of incubation. Uninduced controls [A] and [G]; 6 HAI [B] and [H]; 1 DAI [C] and [I]; 2 DAI [D] and [J]; 4 DAI [E] and [K]; 10 DAI [L]. Note the reduction in shoot meristem size from (G) to (K) and that the SAM is missing in (L) (arrow). The 2 and 3 indicate stage 2 or 3 flower primordia, respectively. Bars = 600 μm for (L) and 100 μm for all other panels. (A) to (E) CLV3 is expressed in the CZ of shoot and floral meristems (arrowheads in [A]). Expression increases dramatically after the inductive treatment. Note that color reactions in (B) to (E) were stopped after 6 h. Inset in (B) shows stage 2 flowering expressing CLV3. (F) Activation of the alcA::GUS reporter gene is revealed by a blue precipitate. Staining is detected in the SAM (asterisk) and floral meristems. (G) WUS RNA is found in the SAM and two floral meristems. (H) to (L) After CLV3 induction, WUS RNA disappears rapidly from the SAM. At 24 HAI (I), only faint or no expression is detected in stage 2 flower meristems (arrowheads). WUS is again expressed in flower meristems at 48 and 96 HAI (insets in [J] and [K]), although CLV3 is also highly expressed at this stage (cf. with (E), stage 2 flower meristem). WUS RNA is present in developing ovules (arrowhead in [L]).

DISCUSSION
We studied the consequences of altering CLV3 expression levels during development for the regulation of meristem size in Arabidopsis. We first found that expression of WUS is repressed rapidly in response to increased CLV3 signaling. CLV3 expression from stem cells is predicted to depend on WUS expression, and consistent with this, we found a strong decrease of CLV3 RNA levels during development and reported that within 24 HAI of CLV3 RNAi, CLV3 expression in the stem cell domain increased (Reddy and Meyerowitz, 2005). We show here that signaling between CLV3 and WUS results in drastic changes in expression levels of both genes within 3 h after altering CLV3 signaling. Although not all components involved in processing of the CLV3 peptide, receptor interaction, and further downstream signaling are known (Trotochaud et al., 1999; Brand et al., 2000; Clark, 2001b; Lenhard and Laux, 2003; Fiers et al., 2005), it is evident that changing the amount of CLV3 mRNA can affect...
signaling intensity through the pathway and therefore ultimately control \( \text{WUS} \) expression in the OC. This indicates that \( \text{WUS} \) expression in the entire OC is accessible for regulation by CLV3 signaling throughout development. The fast feedback regulation of CLV3 expression reveals that \( \text{WUS} \) protein is not long-lasting and that the entire regulatory system is designed to allow for a rapid adjustment of gene expression and cell fate regulation between stem cells and the OC.

We studied the development of individual meristems by scanning electron microscopy analysis of replicas taken at 24-h intervals after CLV3 induction. This showed that \( \text{WUS} \) is required to sustain growth and cell division in the meristem center. It also revealed that primordia initiation is delayed and that the size of newly initiated primordia is increased at the expense of shoot meristem size. Thus, the CZ cells, or \( \text{WUS} \)-expressing cells of the OC, could signal to the periphery to delimit organ primordia initiation. Alternatively, reduction or loss of \( \text{WUS} \) activity from the OC might allow cells to adopt peripheral cell identity as a default cell fate. By downregulating \( \text{WUS} \), growth in the meristem center ceases and the boundary between CZ and PZ identity is shifted, so that the outermost cells of the CZ are now allowed to become incorporated into organ primordia. A converse situation was observed when \( \text{WUS} \) was released from regulation by CLV3, and cells from the PZ were respecified to adopt CZ fate (Reddy and Meyerowitz, 2005). Thus, the balanced signaling between \( \text{WUS} \) and CLV3 is fundamental to positioning of the CZ/PZ boundary.

Judging from our in situ RNA expression analysis, \( \text{WUS} \) is fully repressed in SAMs within 24 HAI. However, most plants receiving only a short induction pulse recovered from CLV3 overexpression that lasted (for a 6-h pulse) \( \sim 30 \) h. To achieve shoot meristem termination in the majority of plants, inductive treatments had to be repeated for \( >3 \) d to maintain high CLV3 levels for at least 96 h. This indicates that \( \text{WUS} \) expression, even if turned off for several days, can be reactivated during an extended period after the initial downregulation. Thus, although \( \text{WUS} \) expression can respond rapidly to alterations of CLV3 signaling, this response may be only transient and not necessarily affect the size of the stem cell population. Importantly, \( \text{WUS} \) expression is governed not only by CLV3 signaling but also by several other control systems (Baurle and Laux, 2005). \( \text{STIMPY} \), encoding a WUS-related homeodomain protein, promotes \( \text{WUS} \) expression in the vegetative apex and can induce overproliferation of stem cells in the absence of CLV signaling (Wu et al., 2005). SPLAYED (SYD), an SNF2-type ATPase, is recruited to the \( \text{WUS} \) promoter and enhances \( \text{WUS} \) transcription within the OC. Genetic analysis predicts that SYD acts independently of CLV to control \( \text{WUS} \) expression (Kwon et al., 2005). Overexpression of miR166g in \( jba-1D \) plants results in increased \( \text{WUS} \) expression. The primary targets for this microRNA are the class III HD-ZIP proteins PHABULOSA (PHB), PHAVOLUTA (PHV), CORONA (CNA), and REVOLUTA (Williams et al., 2005). PHB, PHV, and CNA play redundant roles in modulation of \( \text{WUS} \) expression during development (Green et al., 2005).

### Table 2. Expression of \( \text{WUS} \) in Shoot and Floral Meristems During High CLV3 Expression

<table>
<thead>
<tr>
<th>SAMs</th>
<th>Flower Buds at Stage 2</th>
<th>Flower Buds at Stages 3 to 6</th>
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<tr>
<td>HAI</td>
<td>( n )</td>
<td>With ( \text{WUS} )</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>96</td>
<td>5</td>
<td>0</td>
</tr>
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</table>

\( \text{WUS} \) expression was analyzed by RNA in situ hybridization on tissue sections at given hour after inducing CLV3 (HAI). Flower meristems were staged according to Smyth et al. (1990). \( n \) is the total number of meristems analyzed; the total number of meristems expressing \( \text{WUS} \) and percentage of total are given.

a Unstained bud is at early stage 2.
b Unstained bud is at late stage 6.
c Only weak staining.

**Figure 7.** Activity of CLV3 Promoter Variants and Effects on Meristem Size.

Activity of CLV3 promoter derivatives (V1 to V4) in transgenic plants was measured by a fluorometric GUS assay and is shown as percentage of the activity of a wild-type CLV3 promoter controlling GUS activity (\( p\text{CLV3} = 100\% \)). After replacement of the GUS gene with a CLV3 cDNA, the transgenes were introduced into \( \text{clv3-2} \) mutants. The diameters of the shoot meristems at 10 DAG and carpel numbers were measured and are shown as percentages of wild-type levels (two carpels \( = 100\% \)), n.t., no transgene. Bars indicate standard error; for GUS assays, \( n > 50 \) seedlings; for carpel number, \( n = 50 \); for meristem size, \( n \geq 12 \).
Furthermore, target genes regulated by WUS were found to be protected from CLV3-induced downregulation through sequestration by CLV1 (Lenhard and Laux, 2003). We found that high-level expression of CLV3 can override possible sequestration effects. Furthermore, we did not observe a dramatic rise in CLV1 expression upon ethanol induction that could compensate for the presence of more CLV3 (data not shown).

In both pulsed and continuous CLV3 induction experiments, only a subset of all floral meristems exhibited an effective reduction or loss of stem cells. This was surprising because CLV3 remained expressed at high levels in flower meristems for >7 d in long-term induced plants. WUS expression was initially downregulated in all young floral meristems during the first 24 h after CLV3 induction but became detectable again in most stage 2 flowers later during the inductive period. This apparent adaptation to high-level CLV3 signaling could be explained by the time course of events: ALCR protein has already accumulated in stem cells of young floral meristems before induction and can trigger a rapid and massive increase in CLV3 expression. When new floral meristems are initiated during the inductive treatment, ALCR expression from the (WUS-dependent) CLV3 promoter is feedback regulated and will be expressed at somewhat lower levels. However, ALCR RNA is not rapidly degraded, and we found that even 7 DAI, CLV3 as well as WUS are still expressed at high levels in young floral meristems. Thus, feedback regulation of the inducible transgene cannot significantly contribute to the apparent adaptation during the induction period. Instead, meristems seem to be capable of compensating for excessive CLV3 signaling with time.

Inducible expression of CLV3 using the ALC system always resulted in an extreme (100-fold and more) overexpression of CLV3. However, altering CLV3 expression over a 10-fold range (33 to 300% of wild-type levels) using different promoter variants did not affect the size of shoot or floral meristems. Furthermore, reduced CLV3 expression to 16% wild-type levels was insufficient to fully rescue a clv3 mutant, and both SAM size and carpel cell maintenance.

METHODS

Construction of the iCLV3 Transgenic Plants

To create an ALCR-inducible CLV3 transgene, the CLV3 cDNA was first cloned into pACN1 as a PstI fragment (Salter et al., 1998). The resulting expression cassette (alcA promoter, the CLV3 cDNA and NOS terminator) was cloned into pGPTV-HPT (Becker et al., 1992) to generate pRMALCL (alcA:CLV3). pRMALCL was transformed into Arabidopsis thaliana eco-type Landsberg erecta plants carrying the CLV3:ALCR alcA:GUS transgenes (Deveaux et al., 2003) via the floral dip method (Bechtold and Pelletier, 1998) to give iCLV3. For control experiments, we used the Landsberg erecta acotype, CLV3:ALCR alcA:GUS plants (without alcA: CLV3), and un-induced iCLV3 lines.

Growth Conditions and Ethanol Induction

Plants were grown in Quick-Pot trays at 21 to 28°C, with illumination of 9 Wm⁻². Plants were kept in short days (10-h day/14-h night) for 5 weeks after germination before flowering was induced by transfer to long days (16-h day/8-h night). Induction of CLV3 expression by ethanol vapor (Deveaux et al., 2003) started 4 to 10 d after the transfer to long days, when inflorescence axes were shorter than 7 mm long and the oldest flower bud was still closed (before stage 13 according to Smyth et al., 1990). During inductions, plant trays were covered with transparent hoods.

RNA in Situ Hybridization and GUS Staining

Protocols for GUS stainings, RNA in situ hybridizations, and probe preparation for CLV3 and WUS have been described previously (Brand et al., 2002). As a minor modification, in situ hybridizations and detections were performed with an InSituPro VS robot (Intavis).

Phenotypic Analysis

Photographs were taken with a Canon Powershot G2 digital camera mounted to a Zeiss dissecting microscope, or with an Axioim HR camera attached to a Zeiss Axioscope II microscope. Digital photographs were collated with Adobe Photoshop.

Quantitative Real-Time RT-PCR

For RNA quantifications, total RNA was isolated from seedlings after dissecting leaves and cotyledons using the RNeasy plant mini kit (Qiagen). RNA quality was assayed using the Bioanalyzer 2100 (Agilent). First-strand cDNA synthesis was performed with 5 μg of total RNA using Superscript II RNase H⁻ reverse transcriptase and random oligonucleotide primers (Invitrogen) according to the manufacturer’s instructions. The 20-μL cDNA reaction was diluted 1:75 with deionized water, and 5 μL were used for each RT-PCR amplification. Amplifications were performed as triplicates in 96-well plates in a 25-μL reaction volume containing 12.5 μL 2× Platinum SYBR Green qPCR SuperMix UDG with 0.5 μL ROX dye (Invitrogen). Reactions were performed on a GeneAmp 5700 sequence detection system (Applied Biosystems). For all samples, cDNAs were normalized using Cytochrome B5 isoform 1 (AT5G35360). Primers for all amplifications were located on an exon-exon border to prevent amplification of potentially contaminating genomic DNA. Primers used were as follows: CLV3 (5’-GGACATGA-3’; 5’-CACTTCTCTGCTTCTCCATTTGCTCCAACC-3’), WUS (5’-CAAGTTTCTTTGAGATCGGATAACAG-3’; 5’-CTCTTGGTTTCATTTCTGCT-3’), and Cytochrome B5 (5’-CGACACTGCGAGGACATGA-3’; 5’-ACGTATGTCCTAGTTGCTGGAACA-3’). To distinguish between transcripts from the inducible or the endogenous CLV3 gene,
the following primers were used: inducible CLV3 (3′-primer in the NOS terminator) (5′-TGTGACCCCAAGACAGGCG-3′; 5′-AACGAGAAGCAGGAGTGGAGTGGAGTGGAG-3′), and endogenous CLV3 (5′-primer in the 5′-untranslated region of CLV3) (5′-TCCTCTCACTCAAGTCATTTCTCTCTAA-3′; 5′-AACGAGAAGCAGGAGTGGAGTGGAGTGGAG-3′).

Construction of CLV3 Promoter Variants and GUS Activity Assays

The T-DNA vector pCLV3 (pBU16) that allows GUS expression from the CLV3 promoter has been described previously (Brand et al., 2002). Promoter variants (V) were created by progressively deleting 5′ or 3′ DNA sequences from the CLV3 regulatory sequences in pBU16. Deletions are as follows: V1, 5′ 320 bp and 3′ 660 bp; V2, 5′ 320 bp; V3, 3′ 290 bp; V4, 5′ 1190 bp. Complementation constructs were obtained by cleaving pBU16 with PstI-BamHI and inserting a full-length CLV3 cDNA into these sites. Transformation of ch3-2 mutants and selection of transgenic plants were performed according to previously described procedures (Buchhold and Pelletier, 1998). At least five independent lines were analyzed for each promoter variant.

For quantification of GUS activity, between 20 and 60 10-d-old Arabidopsis seedlings were harvested and analyzed according to published protocols (Weigel and Glazebrook, 2002). Complementation was quantified by counting carpel valve numbers from the first five siliques on 10 plants for each transgenic line. SAM sizes were measured as micrometer surface diameter from cleared seedlings at 10 DAG using Nomarski optics and ImageJ software for image analysis. At least 12 meristems were measured for each genotype.

Quantitative Analysis of Apex Geometry and Growth

In vivo observations and quantitative analysis were performed as described previously (Dumais and Kwiatkowska, 2002; Kwiatkowska, 2004). Briefly, sequences of images showing the surface of individual shoot apices at consecutive instants were obtained using replicas (dental polymer molds) taken from the surface of individual shoot apices. Epoxy resin casts obtained from replicas were analyzed in a scanning electron microscope (LEO435VP; Oxford Instruments). Replicas were taken every 24 h, always from the apex of the main inflorescence axis. Thirteen induced and six control apices were analyzed. Quantitative analysis was performed with the aid of computer programs written in Matlab (The Mathworks). Relative growth rates in area (areal growth rates) for each cell were computed and plotted on the cell wall pattern as it appeared at the beginning of the analyzed time interval. Additionally, cells, which divided during the time interval between successive replicas, were recognized based on the comparison of the cell wall pattern on the apex surface at consecutive instants. Analysis of variance followed by a multiple comparison of means using a Tukey’s honestly significant difference mean-separation test for unequal sample sizes was performed to compare values of SAM and flower primordium areas, numbers of cells counted along the SAM radius, and areal growth rates of cells. Statistica software (Statsoft) was used for this analysis.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative data library under accession numbers At2g27250 (CLV3) and At2g17950 (WUS).

Supplemental Data

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

Supplemental Figure 1. Analysis of Cell Behavior.

Supplemental Figure 2. Meristem Size Measurements.

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Dynamic and Compensatory Responses of *Arabidopsis* Shoot and Floral Meristems to CLV3 Signaling

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**Supplemental Data**
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**References**
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