Stem cell homeostasis in the *Arabidopsis* shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1

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**SUMMARY**

Stem cell maintenance in the *Arabidopsis* shoot meristem is regulated by communication between the apical stem cells and the underlying organizing centre. Expression of the homeobox gene *WUSCHEL* in the organizing centre induces stem cell identity in the overlying neighbours, which then express the *CLAVATA3* gene whose activity in turn restricts the size of the *WUSCHEL* expression domain. We have analyzed how the stem cells and the organizing centre communicate, by studying the mode of action of *CLAVATA3* protein. We provide direct evidence that *CLAVATA3* protein functions as a mobile intercellular signal in the shoot apical meristem that spreads laterally from the stem cells and acts both on their lateral neighbours and on the stem cells themselves to repress *WUSCHEL* transcription. We also show that the spread and range of action of *CLAVATA3* can be limited by binding to its receptor *CLAVATA1*, which offers an explanation for how *CLAVATA3* is prevented from entering the organizing centre and repressing *WUSCHEL* transcription there. This regulated spread of a secreted signalling molecule enables the shoot meristem to permit the onset of cell differentiation in the periphery, but at the same time to maintain a stable niche for its stem cells in the center.

Key words: Stem cells, Shoot meristem, *Arabidopsis thaliana*, CLV3, Ligand sequestration

**INTRODUCTION**

The stem cells in the plant shoot apical meristem (SAM) provide the cells for the continuous formation of aerial organs during postembryonic development (Steeves and Sussex, 1989). The approximately six to nine long-term stem cells are arranged in three tiers and located at the very apex of the SAM. Owing to their preferential planes of division, they give rise to three largely clonally distinct cell layers: stem cells of the two outer tiers divide mainly anticlinally (perpendicular to the surface) and thereby generate the epidermis (L1) and a subepidermal layer (L2), respectively. By contrast, the stem cells below divide both anti- and periclinally (parallel to the surface), giving rise to the interior tissue of the stem and lateral organs (L3).

Stem cell identity appears to be specified by signalling from an underlying cell group, the organizing centre (OC), which expresses the putative homeodomain transcription factor *WUSCHEL* (WUS) (Mayer et al., 1998). Loss of *WUS* function leads to differentiation of stem cells and meristem termination (Laux et al., 1996). By contrast, ectopic *WUS* expression in vegetative organ primordia is sufficient to induce ectopic stem cell identity (Schoof et al., 2000), indicating that *WUS* expression has to be tightly controlled for the SAM to maintain just the right number of stem cells.

The stem cells in turn signal back via the *CLAVATA* (CLV) signalling pathway to restrict the *WUS* expression domain. *clv* mutants develop an enlarged SAM due to the accumulation of stem cells (Clark et al., 1993; Clark et al., 1995; Fletcher et al., 1999; Jeong et al., 1999) and this phenotype is caused by an expansion of the *WUS* expression domain into more apical and peripheral cells (Schoof et al., 2000). *CLV1* encodes a putative leucine-rich repeat transmembrane receptor with an intracellular kinase domain that is expressed in the SAM centre in a region encompassing the OC (Clark et al., 1997). *CLV1* appears to associate with *CLV2*, a similar protein lacking the kinase domain, to form the putative receptor complex for the ligand *CLV3* (Jeong et al., 1999; Trotochaud et al., 1999). *CLV3* codes for a small secreted polypeptide and its expression domain overlaps with the presumed stem cell region (Fletcher et al., 1999; Rojo et al., 2002).

The observations that *WUS* is sufficient to induce expression of *CLV3* as a component of stem cell identity and that *CLV3* acts as a negative regulator of *WUS* expression have led to the proposal that the size of the stem cell population in the SAM is maintained constant by a negative regulatory feedback loop involving the stem cells and the OC (Schoof et al., 2000; Brand et al., 2000).

Although elegant clonal studies using an unstable mutant allele of *clv3* have indicated that *CLV3* activity in the L2 is dispensable for SAM regulation, consistent with a non cell-autonomous function (Fletcher et al., 1999), and Rojo et al. have shown that secretion of *CLV3* protein is required for phenotypic activity in overexpression experiments using the
constitutive Cauliflower Mosaic Virus 35S promoter (Rojo et al., 2002), direct evidence for intercellular movement of CLV3 in the SAM has been lacking to date. In addition, it is not clear where its action is required for stem cell homeostasis. In fact, two observations suggest that CLV3 does not move far away from the stem cells, but acts immediately where it is secreted. Firstly, in clv mutants WUS expression shifts upwards into those cells that in wild type coexpress CLV3 and CLV1, suggesting that CLV3 acts at least in part on the cells secreting it (Schoof et al., 2000). Secondly, ectopic CLV3 expression using the 35S or the UNUSUAL FLORAL ORGANS promoters, both of which have expression domains that most likely encompass the OC (Long and Barton, 1998), causes a wus-like phenotype with meristem termination (Brand et al., 2000), indicating that in wild-type meristems movement of CLV3 protein to the underlying OC cells does not take place.

In this study we address whether CLV3 moves within the SAM, where in the SAM it acts and how it is prevented from repressing WUS expression in the OC. We provide novel evidence that CLV3 protein spreads laterally from the producing stem cells, that this spread is functionally relevant, and that its range of movement can be limited by its receptor CLV1.

MATERIALS AND METHODS

Mutant lines, growth conditions and GUS staining

The wild-type reference used in all experiments was the Landsberg erecta (Ler) ecotype. The intermediate clv-3-1 and strong clv-1-4 loss of function mutants have been described previously (Clark et al., 1993; Clark et al., 1995). Growth conditions and GUS staining were as described previously (Laux et al., 1996; Schoof et al., 2000).

Transgene construction and plant transformation

For all ectopic expression experiments, except for the CLV3::CLV3-GFP and the CLV3::mGFP5-ER lines, we used the pOpL two-component system (Moore et al., 1998). For simplicity, we refer to plants e.g. of the genotype CLV1::LhG4; pOp::CLV3-pOp::GUS as CLV1::CLV3; CLV1::GUS.

Generation of the CLV1::LhG4 and pOp::WUS-pOp::GUS lines has been described previously (Schoof et al., 2000).

For the pOp::CLV3 construct, the CLV3 cDNA was amplified from reverse-transcribed total RNA of wild-type inflorescences using primers CLV3XHO5 (5'-CTC TCG AGT AGT CAC TTG CTC TC-3') and CLV3BAM3 (5'-ACA AGG GAT CCG GTC AAG G-3'), digested with XhoI and BamHI and inserted into MT153 (Lenhard et al., 2002) to yield MT187.

For the pOp::CLV3-pOp::GUS construct, a pOp::GUS fragment was inserted in the unique EcoRI site of MT187 to yield MT204.

For the pOp::CLV3hetSP-pOp::GUS construct, the CLV3 coding sequence lacking the signal peptide was amplified using primers CLV3OHNSP (5'-CTA CTC GAG TGC TTC TTG TTA AAA ATG GAT GC-3') and CLV3BAM3, digested with XhoI and BamHI and inserted into MT204.

For the pOp::CLV3hetSP-pOp::GUS construct, the CLV3 coding sequence lacking its own signal peptide was amplified using primers CLV3-PAP1 (5'-GGG GAG TAT GAT GCT TAT CTA AAT CAT GTC AAC GAC-3') and CLV3BAM3 and the sequence encoding the signal peptide of Purple acid phosphatase1 (GenBank acc.no. U48448) was amplified from genomic DNA using primers PAP1XHO5 (5'-AAC TCG AGT AGT CAC TTG CTC TC-3') and PAP1-CLV3 (5'-GAG ATG AGA AGC ATC GAA CCC TTC ATA GCA AAA CTC-3'). The two PCR products were mixed, allowed to anneal and the fusion gene was amplified using primers PAP1XHO5 and CLV3BAM3. After digestion with XhoI and BamHI it was inserted into MT204.

For the pOp::CLV1 construct, the CLV1 cDNA was amplified from reverse-transcribed total RNA of wild-type inflorescences using primers CLVL1SA5 (5'-TTG TCG AGT AGT CAC TTG CTC TC-3') and CLV1BAM3 (5'-TGG GAT CCT ATT TTA TTG ATC TGG C-3'), digested with SalI and BamHI and inserted into the XhoI and BamHI sites of MT187 to give MT264.

The pOp::clv-1-4 construct was generated by amplifying the region surrounding the clv-1-4 mutation from genomic DNA of homozygous mutants using primers clv1-1-4_P1 (5'-ATT GGA GAT GAA GAG TCT AAC TTT C-3') and clv1-1-4_P2 (5'-TTC CGA GAT TGA AGC TTT GAG-3'), digesting with BpmI and HpaI, inserting this fragment into the CLV1 cDNA, and the modified cDNA then into MT264.

A CLV3-mGFP4 translational fusion was generated by inserting an oligonucleotide encoding five repeats of the dipeptide glycine-alanine (5'-GAT GAT CTT CCT TTT TAA ATG G-3') and CLV3BGL3 (5'-ATT AGA TCT AGG GAT CTT AAA GTT GTC TT-3'), digested with BamHI andBgIII, and inserted into ML360 (ML361). From there, the CLV3-mGFP4 coding sequence was excised as a BamHI-SacI fragment and inserted into the BamHI-SacI sites of the CLV3 promoter present in MT194 (Gross-Hardt et al., 2002).

All fragments amplified by PCR were sequenced to exclude amplification errors.

For the CLV3::mGFP5-ER construct, the mGFP5-ER coding sequence was excised as a BamHI-SacI fragment from pBINPLUS:mGFP5 (kindly provided by J. Haseloff) and inserted into the CLV3 promoter as above.

The pOp::CLV3-mGFP4 construct was generated by excising the CLV3-mGFP4 coding sequence as a BamHI-SacI fragment from ML361, blunt-ending by T4 DNA polymerase and inserting this into the blunt-ended XhoI-BamHI sites of MT204.

For the pOp::CLV3 construct, the pOp::CLV3 cassette was first ligated into the SacI and HindIII sites of pBluescript II SK, from which it was excised using SacI and KpnI, blunt-ended by T4-DNA polymerase and inserted into MT187, which had been digested with HindIII and blunt-ended by T4-DNA polymerase. The resulting plasmid MT274 carries a tandem repeat of the pOp::CLV3 cassette followed by a unique HindIII site. This procedure was repeated until five tandem repeats of the pOp::CLV3 cassette were assembled.

The CLV3::LhG4 construct was generated by inserting the LhG4 coding region as a BamHI-SacI fragment into the CLV3 promoter present in MT194 (Gross-Hardt et al., 2002), replacing the NLSGUS coding region.

Constructs were electroporated into Agrobacterium strain GV3101(pMP90) (Koncz and Schell, 1986) and Ler wild-type plants were transformed by floral dip (Clough and Bent, 1998), unless stated otherwise.

In the progeny of crosses, the presence of the relevant transgenes was monitored either by PCR or by staining for the activity of linked GUS reporters, unless stated otherwise.

For the CLV3::CLV3-GFP; clv3-1/clv3-1 plants, we only analyzed plants heterozygous for the transgene, since, for unknown reasons, homozygosity for the transgene appeared to induce efficient cosuppression: 25% (44 out of 177 plants) of the selfed progeny of pOp::CLV3::LhG4; pOp::CLV3-hetSP-pOp::GUS plants showed a very strong clv3 mutant phenotype and no GFP fluorescence.

In situ hybridization

In situ hybridization using WUS and CLV3 antisense riboprobes has been described previously (Mayer et al., 1998; Schoof et al., 2000).
For the *LhG4* antisense and sense riboprobes, the coding region was inserted into pBluescript, the resulting plasmid linearized and transcribed using T7 and T3 RNA polymerases (Promega), respectively, and digoxigenin RNA labelling mix (Roche Diagnostics). For the *GR* antisense and sense riboprobes, plasmid pRS020 (kindly provided by R. Sablowski) was used. For the *CLV1* antisense and sense riboprobes, the entire cDNA lacking the highly conserved kinase domain encoded within a *XhoI-MunI* fragment was used. BLAST analysis of this sequence against the complete *Arabidopsis* genome did not reveal any sequences with significant homology (not shown).

### GFP imaging

For imaging of GFP fluorescence, inflorescence meristems of transgenic plants were dissected, mounted in 80% glycerol and viewed under a Leica TCS 4D confocal microscope. Images were processed using Adobe Photoshop, version 6.0.

### Scanning electron microscopy and SAM size measurement

For measuring the sizes of inflorescence meristems, plants of the genotypes to be compared were grown in alternate pots within a single tray to ensure equal growth conditions. After the plants had bolted and produced 4–6 siliques, meristems were dissected, fixed and processed for scanning electron microscopy as previously described (Laux et al., 1996). Meristems were photographed from directly above the meristem centre. Sizes were determined on prints of the images by measuring the distance from the centre of the youngest recognisable floral primordium to the centre of the furrow separating the fifth flower primordium from the meristem. This line runs across the centre of the meristem, providing a measure for the meristem diameter. Measurements were taken without knowledge of the genotype of the individual plants to avoid any bias. Statistical analysis was performed using Microsoft Excel, and size distributions were compared by pairwise Student’s *t*-test.

## RESULTS

### Distribution of CLV3-GFP protein in the shoot meristem

We first asked whether the secreted polypeptide CLV3 could move within the SAM and which target cells it could act on. To test this, we generated plants expressing a fusion protein of CLV3 and GFP under the control of the endogenous *CLV3* promoter (see Materials and Methods for a detailed description of expression constructs) and compared the CLV3-GFP protein distribution to the domain of transgene mRNA expression in the SAM of these transgenic plants. This approach was chosen, as the endogenous CLV3 protein could not be detected by immunohistochemistry (data not shown).

We assessed the functionality of the CLV3-GFP fusion protein by transforming the construct into homozygous *clv3-1* mutants and testing for phenotypic rescue. *clv3-1* mutant plants expressing one copy of the transgene had an inflorescence meristem that was only slightly larger than that of the wild type and formed flowers with an average of 3.1 carpels (Fig. 1A,B; Table 1; see Materials and Methods), as compared to a much more enlarged meristem and flowers with 5.4 carpels on average in parallel-grown non-transgenic *clv3-1* mutants (Fig. 1C; Table 1). This degree of rescue provided by the *CLV3::CLV3-GFP* construct was the same as observed in *clv3-1* mutants heterozygous for *CLV3::LhG4* and *pOp::CLV3::pOp::GUS* transgenes that were strongly expressed as judged by staining for the activity of the GUS reporter (3.7 carpels on average; Table 1 and data not shown). This indicates that even though, for unknown reasons, the transgenes appeared to be less effective at restricting meristem size than the endogenous *CLV3* gene of which one copy is sufficient for stem cell homeostasis (Clark et al., 1995), the CLV3-GFP fusion protein had retained CLV3 activity and can therefore be used to monitor functionally relevant protein movement. Importantly, in strong support of this conclusion, the WUS expression domain in *CLV3::CLV3-GFP*; *clv3-1* plants closely resembled that in wild type in contrast to the strongly enlarged domain in non-transgenic *clv3-1* mutants (Fig. 1D–F) (Schoof et al., 2000).

As determined by in situ hybridization using a *mGFP4* antisense riboprobe, the transgene mRNA in *CLV3::CLV3-GFP*; *clv3-1* plants was found solely in a three-to-four-cells high, wedge-shaped domain in the SAM centre with a sharp boundary between expressing and non-expressing cells (Fig. 1J). In line with the somewhat increased SAM size of the rescued mutants, this expression domain was larger than that of the endogenous *CLV3* gene in wild type, yet much smaller than in non-transgenic *clv3* mutants (data not shown) (Fletcher et al., 1999). This mRNA expression domain was compared to the distribution of CLV3-GFP protein as analyzed by confocal microscopy: GFP fluorescence was detectable not only in the region corresponding to the mRNA expression domain, but also in cells towards the periphery of the meristem in the outer layers, extending farthest in the epidermis (Fig. 1K). By contrast, a control construct expressing a cell-autonomous form of GFP (*mGFP5-ER*) from the *CLV3* promoter produced strong GFP fluorescence only in those cells that also expressed the transgene mRNA (Fig. 1G,H). The height of the GFP-positive domain was the same in meristems of the two genotypes (Fig. 1H,K,L), indicating that no detectable levels of CLV3-GFP protein were present in deeper regions of the SAM below the outermost three cell layers.

Thus, even though the CLV3-GFP fusion appears to be somewhat less mobile than the unmodified CLV3 protein (see below), its ability to rescue the mutant phenotype to the same extent as a *CLV3::CLV3* transgene (Fig. 1A–F; Table 1) suggests that the presence of CLV3-GFP protein in cells outside the mRNA expression domain reflects a similar, albeit potentially farther spread of endogenous CLV3 protein.

### Table 1. Carpel numbers of wild-type, *clv3* mutant and transgenic *clv3* mutant plants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean carpel number</th>
<th>s.e.m.</th>
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<tbody>
<tr>
<td><em>CLV3::CLV3</em></td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td><em>clv3-1/clv3-1</em></td>
<td>5.4</td>
<td>0.1</td>
</tr>
<tr>
<td><em>CLV3::CLV3-GFP</em> (heterozygous)</td>
<td>3.1</td>
<td>0.1</td>
</tr>
<tr>
<td><em>CLV3::CLV3</em> (heterozygous)</td>
<td>3.7</td>
<td>0.1</td>
</tr>
<tr>
<td><em>CLV3::CLV3</em> (homozygous)</td>
<td>2.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

For each genotype the first seven flowers of nine individual plants were counted. s.e.m., standard error of the mean.

**CLV3 can act over a distance of several cell diameters**

The above experiment suggests that CLV3 protein can move
within the SAM, either by diffusion or by active transport. However, an alternative mechanism to explain the observed distribution of CLV3-GFP would be inheritance of a stable protein by peripheral stem cell daughters, which themselves no longer express the gene. To distinguish between these possibilities, i.e. protein movement from a stationary source versus clonal inheritance of the protein, we asked whether CLV3 protein could move to and act on cells not clonally related to the producing cells. To test this, we expressed CLV3 specifically in the epidermis of clv3-1 mutants using an ATML1::LhG4 activator (Lu et al., 1996; Sessions et al., 1999) and analyzed whether this would be sufficient to repress WUS in subjacent, clonally unrelated cell layers, thus rescuing the clv3-1 mutant defect. Epidermis-specific expression and transcriptional activation of the ATML1 activator was confirmed by RNA in situ hybridization with LhG4 and GR antisense riboprobes to sections of plants that expressed WUS-GR as an inert reporter mRNA under the control of this activator (Fig. 2A,B).

Surprisingly, ATML1::CLV3 expression in a clv3-1 mutant background not only suppressed the clv3 meristem defect, but caused a wus-like phenotype with termination of seedling meristems and repeatedly initiated adventitious meristems as well as development of flowers lacking the central gynoeicum (Fig. 2E-H). These plants resembled both the wus mutant and the was phenocopy that is produced by CLV3 expression throughout the SAM under the control of the CLV1 promoter (cf. Fig. 5C). The phenotype was dependent on a functional CLV signalling pathway, since ATML1::CLV3 expression had no effect in a clv1-4 mutant background (data not shown), indicating that in the transgenic situation CLV3 acted via its normal downstream pathway.

To test whether this non cell-autonomous action of CLV3 required the protein to be secreted, we repeated the experiment in wild-type background using two modified versions of CLV3 (Fig. 2K), either lacking the signal peptide (CLV3w/oSP) or containing a heterologous signal peptide (CLV3hetSP). For the CLV3hetSP construct, we used the signal peptide from Arabidopsis Purple acid phosphatase1 which is sufficient to target GFP for secretion (Haran et al., 2000) and shows little sequence similarity with the predicted signal peptide of CLV3 (Fig. 2K). While ATML1::CLV3w/oSP expression had no phenotypic effect (Fig. 2J), ATML1::CLV3hetSP expression produced a wus-like phenotype indistinguishable from ATML1::CLV3 (Fig. 2I), indicating that secretion of CLV3 protein from the epidermal cells is required for its effect on the WUS-expressing OC.

Thus, as the epidermal cells are the only source of functional CLV3 protein in ATML1::CLV3; clv3-1 plants, their wus-like phenotype indicates that CLV3 protein produced by epidermal cells is able to act not only in L2 and outer L3 cells, but also in the organizing centre underneath. Although we cannot entirely rule out a relay mechanism in which CLV3 would activate a downstream pathway in L1 cells that would in turn repress WUS in the OC, we consider this unlikely, because the effect of epidermal CLV3 expression shows the same requirements for CLV1 function and for secretion as does its function in stem cell homeostasis. Therefore, the most likely explanation is that CLV3 can move away from the producing cells through clonally unrelated tissue. This in turn suggests that the observed spread of CLV3-GFP fluorescence outside the transgene expression domain in CLV3::CLV3-GFP-expressing plants is largely due to protein movement, and not to inheritance of a stable protein by stem cell daughters.
Fig. 2. Non cell-autonomous effects of CLV3 can be suppressed by CLV1. (A–D) In situ hybridizations to seedling meristems. Control hybridizations using corresponding sense riboprobes did not produce any specific staining (not shown). (A,B) LhG4 (A) and WUS-GR (B) expression in ATML1::LhG4; pOp::WUS-GR plants is restricted to the epidermis of the SAM (arrow) and young leaf primordia. The endogenous WUS gene is expressed in the centre of the SAM in wild-type seedlings, underneath the outermost three cell layers (arrow). (D) Endogenous CLV3 expression is detected in the presumptive stem cells of the SAM in the outermost three cell layers (arrow). (E–J,L–Q) Light micrographs of live plants (E–J,L–O) and GUS-stained, cleared inflorescences (P–Q). (E,F) was-1 mutant (E) and ATML1::CLV3-expressing clv3-1 mutant (F) seedlings 2 weeks after germination. In both cases, the SAM has terminated (arrow) after the formation of two true leaves. (G) Terminated inflorescence of a was-1 mutant plant showing a flower that lacks stamens and carpels (arrow). (H) Inflorescence of an ATML1::CLV3-expressing clv3-1 mutant plant. The meristem has terminated (white arrow) after the formation of several flowers which lack the central gynoecium (black arrow). (I) ATML1::CLV3hetSP-expressing seedling with terminated meristem. (J) ATML1::CLV3w/oSP-expressing seedling. Meristem function is unaffected. (K) Sequence alignment of the translated cDNAs for the endogenous CLV3 (CLV3), the CLV3 gene lacking its signal peptide (CLV3w/oSP) and the CLV3 gene fused to the signal peptide of Purple acid phosphatase1 (CLV3hetSP). Identical amino acids are shaded black, similar amino acids are shaded grey. Note the weak sequence similarity between the endogenous CLV3 and the heterologous Purple acid phosphatase1 signal peptides. The lengths of the predicted signal peptides were determined using TargetP [http://www.cbs.dtu.dk (Emanuelsson et al., 2000)]. The arrow indicates the predicted site of cleavage of the signal peptide for CLV3 and CLV3hetSP. (L) ATML1::CLV3; ATML1::CLV1 coexpressing seedlings are indistinguishable from wild type. (M) ATML1::CLV3; ATML1::clv1-4 coexpressing seedling. The meristem has terminated as in E. (N) Inflorescences of (N) ATML1::CLV3; ATML1::CLV1- and (O) ATML1::CLV3; ATML1::clv1-4-expressing plants. In both cases, the inflorescence meristem is self-maintaining, however, some flowers in O lack a gynoecium (arrow). (P,Q) Inflorescences of ATML1::CLV3 (P) and ATML1::CLV3; ATML1::CLV1 (Q)-expressing plants with strong GUS staining from the ATML1::GUS reporter that is linked to the ATML1::CLV3 gene. (R,S) CLSM images. Signal from GFP fluorescence is shown in green, chlorophyll autofluorescence is in red. (R) ATML1::CLV3-GFP plant with an even gradient of fluorescence extending from the epidermis to the centre of the meristem. (S) ATML1::CLV3-GFP; ATML1::CLV1 coexpressing plant with strong fluorescence in the epidermis of the inflorescence meristem, yet only very weak signal in the underlying cell layer. Note that in (R) and (S), strong GFP fluorescence is only visible in shoot and floral meristems, even though the ATML1 activator is expressed in the epidermis throughout the aerial part of the plant (compare with P,Q). This lack of a signal could either be due to weaker ATML1 promoter activity or to a post-transcriptional regulation of CLV3 expression outside of the SAM. Scale bars: 50 µm (A–D,R,S); 1 mm (E–J,L–Q).
CLV signalling in stem cell neighbours is essential for meristem homeostasis

We next asked whether this apparent movement of CLV3 and its action on non-expressing cells is necessary for normal SAM regulation, or whether autocrine action of CLV3 only on the secreting cells is sufficient for stem cell homeostasis. To study this, we generated plants in which only the CLV3-expressing stem cells have a functional CLV1 receptor by crossing a CLV3::CLV1 transgene, which did not have any phenotypic effects in wild type (Table 2 and data not shown), with CLV1::CLV1 expressing clv1-4 mutant plants, respectively. The meristem size of the CLV3::CLV1-expressing clv1-4 mutant is intermediate between the wild type and CLV1::CLV1-expressing clv1-4 mutant plants, on the one hand and the enlarged clv1-4 mutant meristem on the other. IM, inflorescence meristem. Scale bars: 50 μm (in A for A and B and C for C-F).

The range of CLV3 action can be restricted by the CLV1 receptor

The above results raised a paradox: ectopically expressed CLV3 from the epidermis could not cell-autonomously repress WUS in the OC. However, in the wild-type SAM WUS expression in the OC is not affected by CLV3 expression in the stem cells immediately above (Fig. 2C,D). A conceivable mechanism, consistent with the localization of the CLV3-GFP protein (see above), could be that in the wild type most CLV3 protein is bound by the putative CLV1 receptor of cells in the L3 and possibly also the L2 layers (Clark et al., 1997) and thus cannot spread into underlying OC cells. By contrast, in ATML1::CLV3-expressing plants this hypothetical block to CLV3 movement would then be predicted to be ineffective, because an excess of CLV3 protein is secreted by the epidermal cells that cannot fully be bound by CLV1, allowing CLV3 protein to reach the OC. To test whether such a mechanism of ligand sequestration is functional in the SAM, we compared the effects of expressing CLV3 alone to those of coexpressing CLV3 and CLV1 under the control of the ATML1 promoter in a wild-type background. If CLV1 protein was able to keep CLV3 from moving away from the producing cells, this would be predicted to suppress the meristem termination caused by ATML1::CLV3 expression.

For each genotype the first seven flowers of four individual plants were counted.

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<th>Genotype</th>
<th>Mean carpel number</th>
<th>s.e.m.</th>
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<tbody>
<tr>
<td>CLV1/CLV1</td>
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<td>0.1</td>
</tr>
<tr>
<td>CLV3::CLV1; clv1-4/clv1-4*</td>
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<td>0.1</td>
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*Only plants with the modified phenotype are included here (see text for details). s.e.m., standard error of the mean.

For each genotype the first seven flowers of four individual plants were counted.
We crossed homozygous ATML1::LhG4 plants to plants that were heterozygous for both a pOp::CLV3-pOp::GUS and an unlinked pOp::CLV1 transgene and analyzed the F1 progeny for meristem defects. In contrast to the 50% of wus phenocopies expected if CLV1 coexpression had no effect, only 27.8% (88 out of 317) of the seedlings showed a wus-like phenotype, while 72.2% (229) were indistinguishable from wild type with a functional, self-maintaining meristem.

Doubly transgenic plants coexpressing CLV1 and CLV3 were identified by PCR (data not shown) and were indeed indistinguishable from wild type at the seedling stage (Fig. 2L). After bolting, they exhibited indeterminate growth of the inflorescence meristem and formed complete flowers like those of the wild type (Fig. 2N). The phenotypic rescue was not complete, however, as they occasionally failed to form meristems in the axils of cauline leaves (data not shown). The integrity of the CLV3 transgene in these rescued ATML1::CLV3; ATML1::CLV1 plants was confirmed by analyzing their progeny, which again segregated for wus-like phenotypes (20 out of 88 seedlings analyzed). In addition, staining for the activity of the GUS reporter that is linked to the CLV3 transgene (Fig. 2P,Q) demonstrated that CLV3 expression domain compared to wild type in 6 out of 9

Table 3). As shown by in situ hybridization using a detailed analysis, we measured the meristem sizes of CLV3-overexpressing plants (genotype CLV3::LhG4; (pOp::CLV3); pOp::GUS) as compared to CLV3::GUS control plants by scanning electron microscopy (Fig. 4E,F). The average meristem size of CLV3::(CLV3)5-expressing plants was reduced by more than 20% compared to the control (42.5 μm versus 54.2 μm; Table 3). This difference was statistically highly significant, as indicated by Student’s t-test (P<0.01; Table 3). As shown by in situ hybridization using a WUS antisense riboprobe, the decreased meristem size of CLV3-overproducing plants correlated with a narrower WUS expression domain compared to wild type in 6 out of 9 CLV3::(CLV3)5-expressing plants analyzed (Fig. 4C,D).

Thus, overproduction of CLV3 by the stem cells reduces the CLV3 in shoot meristem regulation
size of the meristem, presumably by decreasing the size of the WUS-expressing organizing centre.

The reduction of meristem size in this experiment could either be due to the expansion of the CLV3 mRNA expression domain to lateral and basal stem cell daughters (see above) or to more CLV3 protein moving from the secreting cells to their neighbours. We sought to distinguish between these possibilities by coexpression of CLV1 under the control of the CLV3 promoter: if more CLV3 protein moving from the stem cells to their neighbours caused the smaller meristems, coexpression of CLV3::CLV1 should be able to suppress the effect by binding more CLV3 protein in or on the secreting cells, and thus blocking its movement.

We measured the meristem sizes of CLV3::(CLV3)5; CLV3::CLV1-expressing plants in the same experiment as above. Their meristems were approximately 20% larger than those of CLV3::(CLV3)5-expressing plants (51.3 μm versus 42.5 μm; P<0.001; Table 3), yet still slightly smaller than those of CLV3::GUS controls (P<0.01). In situ hybridization using a CLV1 antisense riboprobe suggested that the increase in SAM size compared to CLV3::(CLV3)5-expressing plants was not due to cosuppression of CLV1 and therefore reduced sensitivity to CLV3 (data not shown). As the number of pOp promoters was kept constant between the two genotypes (CLV3::LhG4; (pOp::CLV3)5; pOp::GUS versus CLV3::LhG4; (pOp::CLV3)5; pOp::CLV1), the effect was not due to weaker expression of the CLV3 transgenes because of competition by the pOp promoters for the LhG4 transcription factor; it was more probably due to CLV3 binding to CLV1 in or on the stem cells. This in turn suggests that most of the reduction in meristem size by CLV3 overproduction was due to more CLV3 protein moving away from the stem cells.

In summary, the size of the SAM can be reduced by overproduction of CLV3 in the stem cells, with the magnitude of the effect depending on the amount of CLV3 protein that is free to move away from the stem cells. However, the WUS-CLV3 feedback system appears to be sufficiently buffered to prevent meristem termination even with five extra copies of CLV3 present.

The CLV signalling pathway represses transcription from the WUS promoter

In a last experiment, we asked what the consequences of perceiving the CLV3 signal are in the target cells. Signalling by CLV3 through the CLV1 receptor is thought to activate an intracellular phosphorylation pathway (Trotochaud et al., 1999) which represses WUS expression (Brand et al., 2000; Schoof et al., 2000). Previous work had not addressed the question of whether it does so by repressing WUS transcription or whether it acts at a posttranscriptional level, e.g. by influencing mRNA stability. To distinguish between these possibilities, we expressed either CLV3 alone or both CLV3 and the WUS cDNA (containing the 5′ UTR except for the first six nucleotides and the entire 3′ UTR, but lacking the two introns), under the control of the CLV1 promoter. If the CLV pathway mainly represses transcription from the WUS promoter, coexpression of WUS from the heterologous CLV1 promoter would be predicted to be dominant over the effect of ectopic CLV3 expression. By contrast, if CLV signalling affects WUS activity at some posttranscriptional level, it should still do so when the WUS mRNA is expressed from a heterologous promoter.

In the progeny of a control cross of homozygous CLV1::LhG4 plants to heterozygous pOp::CLV3 plants, 32 out of 65 seedlings (49.2%) were indistinguishable from wus
DISCUSSION

The size of the stem cell population in the Arabidopsis shoot meristem is regulated by a negative feedback loop between the stem cells and the cells of the underlying OC, mediated by the WUS and CLV3 genes (Brand et al., 2000; Schoof et al., 2000). WUS signalling from the OC specifies the overlying neighbours as stem cells and induces the expression of CLV3 which in turn restricts the WUS expression domain. By this mechanism, the stem cell population can be kept constant despite transient fluctuations, e.g. in cell division rates.

Previous work had demonstrated that CLV3 encodes a secreted polypeptide which acts in the extracellular space (Rojo et al., 2002). It was not known, however, whether CLV3 moves within the SAM, which cells it acts on, and how its spread is limited to prevent repression of WUS expression in the OC. Here we have addressed these questions about how CLV3 protein acts in the communication between stem cells and their neighbours.

A model for communication between the stem cells and their neighbours

Our results provide direct evidence that CLV3 protein can spread from the producing cells to their neighbours and repress WUS expression there, and that this action on neighbouring cells is necessary for stem cell homeostasis. Non cell-autonomous effects of CLV3 can be abolished by coexpression of CLV1, which appears to bind CLV3 on the producing cells, limiting its movement.

Our results suggest the following model for how the stem cells interact with their neighbours to maintain a constant stem cell population (Fig. 6). The stem cells secrete CLV3 protein, some of which moves to neighbouring cells. CLV3 acts both on the stem cells themselves and on their neighbours to repress transcription from the WUS promoter. By restricting the stem cell inducing signal from the OC in this manner, the stem cells exert an indirect lateral inhibition on their daughters, allowing these to initiate differentiation. Which cells CLV3 can reach is determined by the expression of its receptor CLV1, as CLV1 sequesters the ligand and prevents further movement. The strong CLV1 expression in the meristem centre restricts movement of CLV3 from the stem cells downwards, while lateral movement can occur in the outer layers in which there is little or no CLV1 protein. Thus, CLV1 protects the OC from CLV3 entering it and allows WUS expression there, ensuring continued stem cell and meristem activity.

CLV3 as an intercellular signal in the SAM

To test where CLV3 protein is localized in the SAM and which cells it can act on, we have used a CLV3-GFP fusion protein expressed under the control of the CLV3 promoter. The ability of the CLV3-GFP construct to rescue the clv3 mutant phenotype as efficiently as a CLV3 transgene suggests that the endogenous CLV3 protein, although we cannot exclude that endogenous CLV3 may spread farther: GFP fluorescence was found extending from the stem cells to the SAM periphery in a cap that encompassed the epidermis and two subepidermal layers. However, no fluorescence was detectable in SAM cells below the apical stem cells.

The presence of CLV3 mainly in the stem cells and their lateral neighbours is also supported by functional data concerning its primary site of action: CLV signalling exclusively in the stem cells themselves could only partially rescue the clv mutant phenotype, indicating that CLV3 acts on
the producing stem cells themselves, but must also be perceived in stem cell neighbours for proper SAM regulation.

Taken together, these data suggest that CLV3 spreads laterally from the stem cells and acts both in these lateral neighbours and in the L2 and L3 stem cells themselves to repress WUS transcription (Fig. 6).

**Maintaining a stable OC**

Ectopic expression of CLV3 throughout the SAM causes termination of stem cell maintenance by repressing WUS expression in the OC. As a consequence, the stem cells would threaten their own existence, if the range of CLV3 action were not restricted to keep CLV3 out of the OC. Based on our results, this restriction of CLV3 movement can be achieved by binding of CLV3 to its putative receptor CLV1 in outermost L3 and possibly also L2 cells. According to this view, CLV1 would fulfil a dual function: it relays the CLV3-dependent signal into the receiving cells and ultimately causes repression of WUS transcription in apical cell layers. By sequestering the ligand, at the same time it also protects the underlying cells of the OC from CLV3 and thus allows WUS expression there.

In support of this interpretation, CLV1 expression from the ATML1 or CLV3 promoters could clearly suppress non cell-autonomous effects of the respective transgenic CLV3 expression. However, it had no phenotypic effects in wild type. A possible explanation for this discrepancy could be that even in the presence of additional CLV1 protein in wild-type background sufficient CLV3 ligand can still move to lateral neighbours to ensure stem cell homeostasis. By contrast, because of the strong endogenous CLV1 expression in the L3, even in CLV3:(CLV3)3-expressing plants only small amounts of CLV3 protein may reach the OC, causing the reduction in SAM size, and this appears to be effectively inhibited by additional CLV1 expression in the CLV3-secreting cells. Clearly, a rigorous test for the importance of the proposed mechanism of ligand sequestration in wild-type meristem regulation has to await further experiments.

**Regulation of meristem size and shape**

The SAM represents a stem cell system that functions over a long period of time with varying degrees of activity, e.g. dormancy in winter and reactivation in the subsequent spring. Thus, continuous SAM function likely requires that meristem organization be buffered against external fluctuations and disturbances. An important mechanism to achieve this seems to be provided by the autoregulatory interaction between the stem cells and the OC, mediated by the WUS and CLV3 genes (Schoof et al., 2000). The robust homeostatic potential of this interaction to keep the size of the stem cell population constant is highlighted by the striking difference in the effects of CLV3 transgenes, depending on the promoter used: one copy of an ATML1::CLV3 transgene was sufficient to cause SAM termination, whereas up to five additional copies of CLV3 could be tolerated and merely caused a reduction in size of the meristem, when expressed under the control of the CLV3 promoter. As the ATML1 promoter is independent of WUS activity (cf. Fig. 2P), the amount of CLV3 secreted from the epidermis can apparently overcome the supposed block to CLV3 movement into the OC imposed by the CLV1 receptor and repress WUS transcription there. By contrast, as the CLV3 promoter depends on WUS activity (Brand et al., 2002; Lenhard et al., 2002), downregulation of WUS expression in CLV3:(CLV3)3-expressing plants also causes a reduction in expression levels of the transgene, allowing a new balance between WUS and CLV3 activities to be struck and thus the stable maintenance of a smaller meristem. Thus, changing the activity of one of the interactors in the WUS-CLV3 feedback loop does not result in gross defects, but rather only shifts the point of equilibrium with respect to stem cell number.

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