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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* (L*er*) ecotype. The intermediate *clv3-1* and strong *clv1-4* 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 AGC AGT CAC TTT CTC TC-3′) and CLV3BAM3 (5′-ACA AGG GAT CCG GTC AAG GG-3′), digested with *Xho*I and *Bam*HI and inserted into MT153 (Lenhard et al., 2002) to yield MT187.

For the *pOp::CLV3-pOp::GUS* construct, a *pOp::GUS* fragment was inserted into the unique *Eco*RI site of MT187 to yield MT204.

For the *pOp::CLV3w/oSP-pOp::GUS* construct, the *CLV3* coding sequence lacking the signal peptide was amplified using primers CLV3OHNESP (5′-CTA CTC GAG TGC TTC TTG TTC AAA ATG GAT GC-3′) and CLV3BAM3, digested with *Xho*I and *Bam*HI 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′-GGA GGG TTC GAT GCT TCT GAT CTC AC-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 AGA AAC TAA TCT TGA AGA TGA G-3′) and PAP1-CLV3 (5′-GAG ATC AGA AGC ATC GAA CCC TCC 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 *Xho*I and *Bam*HI 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 CLV1SAL5 (5′- TTG TCG ACC GCT CTT TCT CAC TGA GAG C-3′) and CLV1BAM3 (5′-TCG GAT CCT ATT TTC ATA TTT ATC TTG C-3′), digested with *Sal*I and *Bam*HI and inserted into the *Xho*I and *Bam*HI sites of MT187 to give MT264.

The *pOp::clv1-4* construct was generated by amplifying the region surrounding the *clv1-4* mutation from genomic DNA of homozygous mutants using primers clv1-4_P1 (5′-ATT GGA GAT GAA GAG TCT AAC TTC TCT C-3′) and clv1-4_P2R (5′-TTC CGA GAT TGA TGA AGC TTT GAG-3′), digesting with *Bpm*I and *Hpa*I, 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 CCG GTG CAG GAG CTG GCG CCG GCG CAG GTG CGA TC-3′) into the *Bam*HI site 5′ of the *mGFP4* coding sequence in pUC19 (Haseloff et al., 1997) to give ML360. The *CLV3* coding sequence was amplified using primers CLV3BAM5 (5′-GAC GGA TCC CTT TCT CTC TAA AAA ATG G-3′) and CLV3BGL3 (5′-ATT AGA TCT AGG GAG CTG AAA GTT GTT TC-3′), digested with *Bam*HI and *Bgl*II, and inserted into ML360 (ML361). From there, the *CLV3-mGFP4* coding sequence was excised as a *Bam*HI-*Sac*I fragment and inserted into the *Bam*HI-*Sac*I 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 *Bam*HI-*Sac*I 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 *Bam*HI-*Sac*I fragment from ML361, blunt-ending by T4 DNA polymerase and inserting this into the blunt-ended *Xho*I-*Bam*HI sites of MT204.

For the *pOp::(CLV3)*$_2$ construct, the *pOp::CLV3* cassette was first ligated into the *Sac*I and *Hin*dIII sites of pBluescript II SK, from which it was excised using *Sac*I and *Kpn*I, blunt-ended by T4-DNA polymerase and inserted into MT187, which had been digested with *Hin*dIII and blunt-ended by T4-DNA polymerase. The resulting plasmid MT274 carries a tandem repeat of the *pOp::CLV3* cassette followed by a unique *Hin*dIII 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 *Bam*HI-*Sac*I 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 L*er* 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 four independent primary transformants that were all PCR-positive for the transgene 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 *Xho*I-*Mun*I 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).

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

Genotype	Mean carpel number	s.e.m.
CLV3/CLV3	2.0	0.0
clv3-1/clv3-1	5.4	0.1
CLV3::CLV3-GFP (heterozygous)*; clv3-1/clv3-1*	3.1	0.1
CLV3::CLV3 (heterozygous)*; clv3-1/clv3-1*	3.7	0.1
CLV3::CLV3 (homozygous)*; clv3-1/clv3-1*	2.1	0.1

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

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 *clv* 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.

CLV3 can act over a distance of several cell diameters

The above experiment suggests that CLV3 protein can move

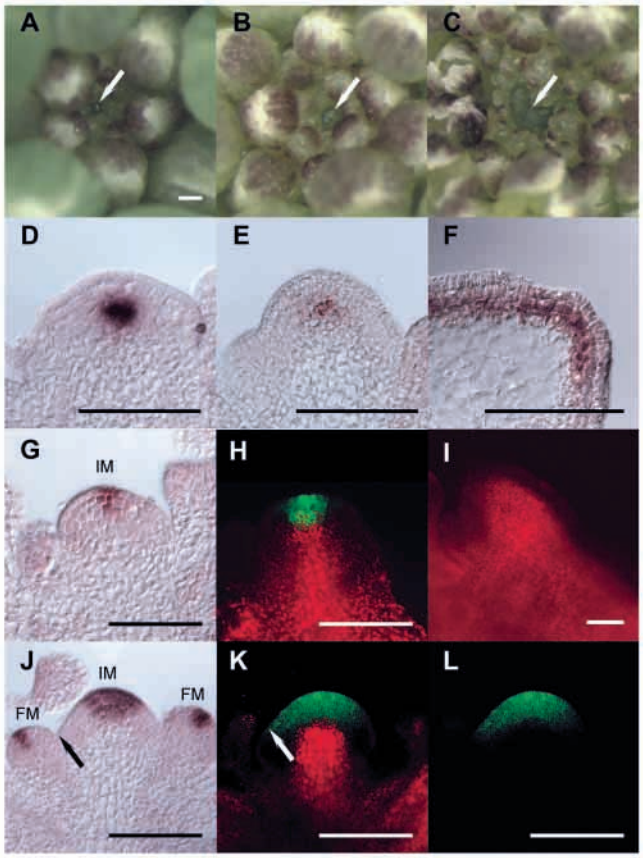

Fig. 1. CLV3-GFP protein localization. (A-C) Photographs of live inflorescences. Arrows indicate the inflorescence meristem. (A) Wild-type inflorescence. (B) *CLV3::CLV3-GFP*-expressing *clv3-1* mutant. The meristem is only slightly larger than in A. (C) Non-transgenic *clv3-1* mutant. The meristem is grossly enlarged. (D-F,G,J) In situ hybridizations with *WUS* (D-F) and *GFP* (G,J) antisense probes. Control hybridizations using corresponding sense riboprobes did not produce any specific staining (not shown). (H,I,K,L) CLSM images. GFP fluorescence is shown in green, chlorophyll autofluorescence is in red. (D-F) *WUS* expression in *CLV3::CLV3-GFP*-expressing *clv3-1* mutant plants is restricted to a small group of cells underneath the presumed stem cells (E), as it is in wild type (D). By contrast, the expression domain is greatly enlarged in non-transgenic *clv3-1* mutants (F). (G,H) In *CLV3::mGFP5-ER*-expressing wild-type background, strong GFP fluorescence is restricted to the apical stem cells (H) that also show *GFP* mRNA expression (G). (I) Non-transgenic *clv3-1* mutant inflorescence imaged under the same conditions as K does not show any fluorescence in the GFP channel. (J-L) In *CLV3::CLV3-GFP*-expressing *clv3-1* mutant plants, GFP fluorescence (K,L) is detectable outside the domain of *GFP* mRNA expression (J), extending towards the meristem periphery. Arrows in J and K indicate the boundary between the inflorescence meristem and comparable young flower meristems. (L) Same apex as in K showing only GFP fluorescence. Note the spread of the signal laterally, but not into deeper regions of the SAM. IM, inflorescence meristem; FM, floral meristem. Scale bars: 100 μm in A (for A-C), 50 μm in D-L.

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 gynoecium (Fig. 2E-H). These plants resembled both the *wus* mutant and the *wus* 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.

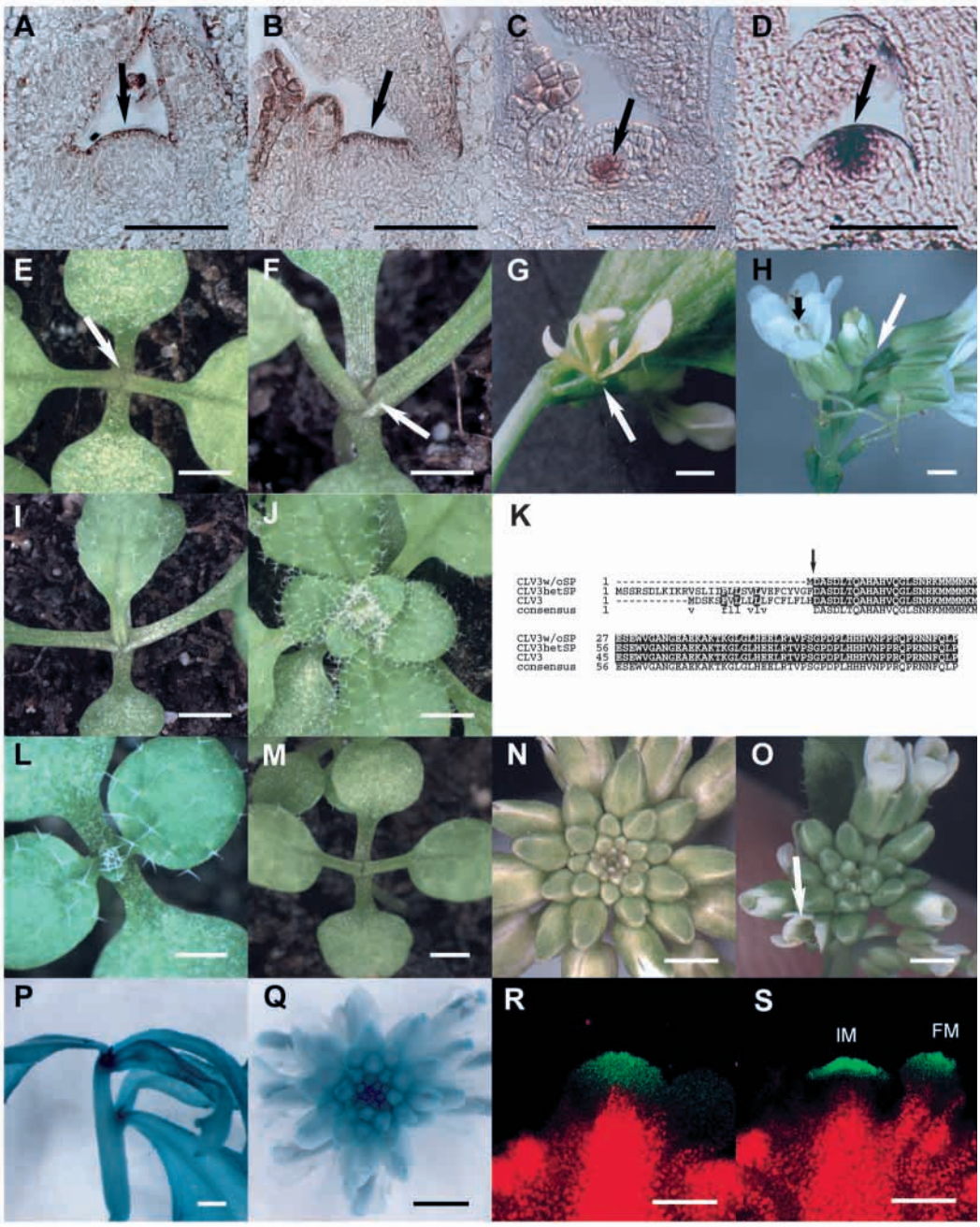

Fig. 2. Non cell-autonomous effects of CLV3 can be suppressed by CLV1. (A-D) In situ hybridizations to seedling meristems. Control hybridizations using corresponding sense riboprobes did not produce any specific staining (not shown). (A,B) *LhG4* (A) and *WUSGR* (B) expression in *ATML1::LhG4; pOp::WUS-GR* plants is restricted to the epidermis of the SAM (arrow) and young leaf primordia. (C) 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 presumed 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) *wus-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 *wus-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,O) 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).

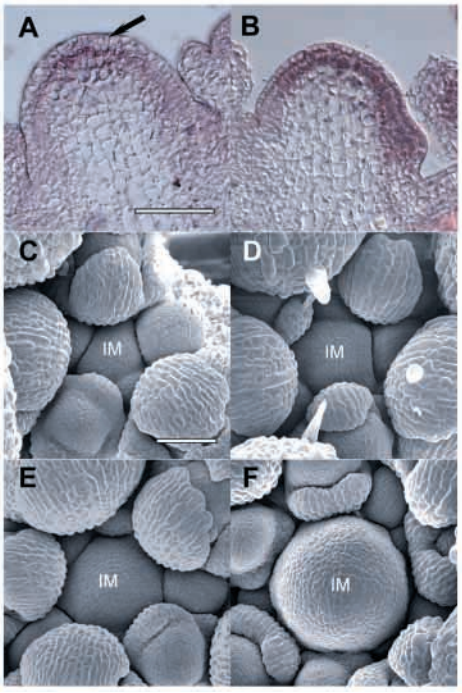

Fig. 3. Perception of CLV3 in stem cell neighbours is required for meristem regulation. (A,B) *CLV1* expression as detected by RNA in situ hybridization in non-transgenic *clv1-4* mutant (A) and *CLV3::CLV1; clv1-4* (B) plants. Note expression in the epidermis in B which is not present in A (arrow). Hybridization with a sense probe did not produce any specific staining (not shown). (C-F) Scanning electron micrographs of secondary inflorescence meristems of wild type (C) and *CLV1::CLV1*-expressing (D), *CLV3::CLV1*-expressing (E) and non-transgenic (F) *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).

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), into a *clv1-4* mutant background. In situ hybridization to sections of *CLV3::CLV1* transgenic *clv1-4* mutants using a *CLV1* antisense probe produced an ectopic signal in the epidermis in addition to the endogenous expression in subepidermal cells, suggesting that the transgene was expressed (Fig. 3A,B). *CLV3::CLV1* expression produced only a partial rescue of the *clv1-4* mutant phenotype: 20 out of 36 *CLV3::CLV1*-expressing *clv1-4* mutants showed an unmodified *clv1-4* mutant phenotype with a strongly enlarged SAM and supernumerary carpels, whereas the remaining 16 plants exhibited a phenotype intermediate between wild-type and non-transgenic *clv1-4* mutants, with regard to the size of the inflorescence meristem and carpel numbers (Fig. 3C-F; Table 2).

Table 2. Carpel numbers of wild-type, *clv1* mutant and transgenic *clv1* mutant plants

Genotype	Mean carpel number	s.e.m.
CLV1/CLV1	2.0	0
clv1-4/clv1-4	6.6	0.1
CLV1::CLV1; clv1-4/clv1-4	2.3	0.1
*CLV3::CLV1; clv1-4/clv1-4**	4.0	0.1

For each genotype the first seven flowers of four individual plants were counted.
*Only plants with the modified phenotype are included here (see text for details). s.e.m., standard error of the mean.

This only modest effect of the *CLV3::CLV1* transgene contrasted with the ability of a *CLV1::CLV1* transgene – using the same *pOp::CLV1* line – to almost fully complement the *clv1-4* defect (Fig. 3C,D; Table 2). It appears unlikely that the weaker effect of the *CLV3::CLV1* transgene is simply due to a lower expression level than that of the *CLV1::CLV1* transgene, because control experiments with a GUS reporter indicated that expression controlled by the *CLV3* activator was at least as strong as that produced by the *CLV1* activator (data not shown).

Thus, the partial rescue in almost half of the *CLV3::CLV1*-expressing *clv1-4* mutants suggests that CLV3 protein exerts an autocrine effect on the stem cells themselves. However, as the rescue is not complete, it appears that in addition CLV3 action is required in stem cell neighbours for proper SAM regulation.

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 non 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.

First, as expected 100% (n=182) of *ATML1::CLV3*-expressing plants phenocopied the *wus* mutant, indicating that the *ATML1::CLV3* effect is fully penetrant. By contrast, *ATML1::CLV1* expression on its own did not have any obvious morphological effects (data not shown).

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 F_1 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 the suppression of the *ATML1::CLV3* phenotype by coexpression of *CLV1* was not due to downregulation of the *ATML1* promoter. This suggests that coexpression of *CLV1* suppresses the long-range effects of CLV3 over several cell diameters.

To test whether this activity of CLV1 correlated with its ability to bind CLV3, we asked whether clv1-4, a mutant form of CLV1 that forms a less stable receptor complex and is therefore predicted to bind less CLV3 protein (Trotochaud et al., 1999) would be impaired in suppressing its long-range action. We found that *ATML1::CLV3; ATML1::clv1-4* coexpressing plants were indistinguishable from *wus* mutants at the seedling stage (Fig. 2M), indicating that clv1-4 protein was less effective than wild-type CLV1 in restricting the range of CLV3. After the floral transition *ATML1::CLV3; ATML1::clv1-4* coexpressing plants formed a flowering shoot with a self-maintaining inflorescence meristem, yet no axillary meristems were present in the axils of cauline leaves, and several flowers lacked a gynoecium (Fig. 2O).

Thus, in contrast to wild-type CLV1, the clv1-4 protein could only partially suppress the long-range action of CLV3. This suggests that CLV1 represses non cell-autonomous effects of CLV3 by ligand sequestration and therefore that the protein distribution of CLV3 was altered by coexpressed CLV1. To demonstrate this directly, we expressed either CLV3-GFP alone or in combination with CLV1 in the epidermis.

The distribution of GFP fluorescence in the meristems of *ATML1::CLV3-GFP*- and *ATML1::CLV3-GFP; ATML1::CLV1*-expressing plants was compared by confocal microscopy. In plants expressing only CLV3-GFP, 76% (16 out of 21) of the inflorescence meristems analyzed showed an even gradient of fluorescence extending from the epidermis into deeper regions of the SAM (Fig. 2R), whereas the remaining 24% of SAM preparations had GFP fluorescence mainly in the epidermis. By contrast, consistent with the previous functional data, 78% (18 out of 23) of the meristems from plants coexpressing CLV3-GFP and CLV1 in the epidermis had strong GFP fluorescence restricted to the epidermis and much weaker fluorescence in the underlying cells (Fig. 2S), whereas the remaining meristems showed a more even distribution extending into deeper regions of the SAM. Although the reason for this failure of CLV1 to prevent movement of CLV3-GFP in all cases is unclear, a Chi-square test indicated that the difference between *ATML1::CLV3-GFP* and *ATML1::CLV3-GFP; ATML1::CLV1*-expressing plants was statistically highly significant ($P<0.01$). These results demonstrate that coexpressed CLV1 can restrict the movement of the CLV3-GFP protein.

In summary, these results show that coexpression of CLV1 is able to suppress non cell-autonomous effects of CLV3 presumably by preventing CLV3 movement away from producing cells. In addition, they also support the interpretation that the *wus* phenocopy produced by *ATML1::CLV3* expression is due to CLV3 protein movement to the OC and not to a relay mechanism, as the latter would be expected to work more, and not less effectively when the CLV1 receptor is coexpressed.

We note that while the CLV3-GFP fusion protein rescued the *clv3-1* mutant defect to the same extent as unmodified CLV3, its expression in the epidermis did not cause meristem termination as observed in *ATML1::CLV3* plants (data not shown). This discrepancy can be explained by assuming that for stem cell regulation only a relatively small amount of CLV3 is required to reach the neighbouring cells, which can apparently still be achieved even by a less mobile CLV3-GFP fusion. By contrast, in order for CLV3 protein, secreted from the L1, to repress *WUS* in the more distant OC, a more extensive CLV3 protein movement seems to be required than can be achieved by CLV3-GFP.

Increasing the CLV3 dosage reduces meristem size

The above ectopic expression experiments suggested that the range of CLV3 action is sensitive to the ratio of CLV1 to CLV3 doses. To test whether the relative levels of CLV3 and CLV1 expression affect stem cell homeostasis, we asked whether overproduction of CLV3 from the stem cells could influence the OC and reduce meristem size or even cause meristem termination, or whether the feedback system between stem cells and OC is buffered. To test this, we analyzed the meristem phenotype of plants that contained up to five additional copies of *CLV3* (see Materials and Methods). In situ hybridization using a *CLV3* antisense riboprobe on sections of *CLV3::(CLV3)$_5$*-expressing plants showed strong staining restricted to the stem cell region in the outermost three cell layers of the inflorescence meristem, similar to wild type (Fig. 4A,B), with, however, weak but clear staining also in the immediate lateral and basal stem cell daughters.

Plants with extra *CLV3* copies did not show any gross developmental alterations (data not shown). For a more detailed analysis, we measured the meristem sizes of *CLV3*-overexpressing plants (genotype *CLV3::LhG4; (pOp::CLV3)$_5$; 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 ($P<0.001$; 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

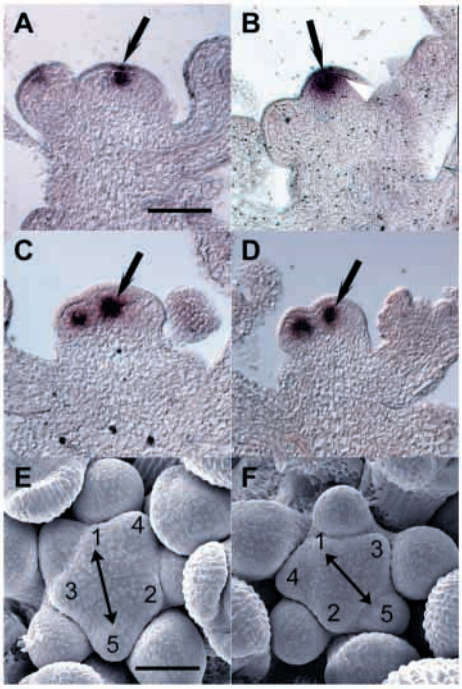

Fig. 4. Reduction of SAM size by CLV3 overproduction. (A-D) In situ hybridizations to inflorescences of wild type (A,C) and *CLV3::(CLV3)$_5$*-expressing plants (B,D). Control hybridizations using corresponding sense riboprobes did not produce any specific staining (not shown). (A) In the wild-type inflorescence meristem *CLV3* expression is restricted to the presumed stem cells in the centre of the three outermost cell layers (arrow). (B) In inflorescence meristems of *CLV3::(CLV3)$_5$*-expressing plants strong hybridization signal with the *CLV3* antisense riboprobe is detected in the presumed stem cells, similar to wild type (arrow). However, weak staining is also observed in lateral and basal stem cell daughters (arrowhead). (C,D) *WUS* is expressed in the centre of wild-type (C) and *CLV3::(CLV3)$_5$*-expressing (D) inflorescence meristems underneath the presumed stem cells. However, the width of the expression domain appears to be reduced in D compared with C. (E,F) Scanning electron micrographs of *CLV3::GUS*- (E) and *CLV3::(CLV3)$_5$*-expressing (F) plants. Young flower primordia are numbered successively 1 to 5, and the distance measured for determining meristem size is indicated by double-headed arrows. Scale bars: 50 μm (in A for A-D and E for E,F).

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.

Table 3. Inflorescence meristem sizes

Genotype	Mean inflorescence width (μm)	s.e.m. (μm)	*n*
*CLV3:: GUS**	54.2	0.7	44
CLV3::(CLV3)$_5$; CLV3::GUS	42.5	0.7	37
*CLV3::(CLV3)$_5$; CLV3::CLV1**,†	51.3	0.8	28
*CLV3::CLV1**	54.8	0.6	31

The sizes of inflorescence meristems of plants with the indicated genotypes were measured as described in Materials and Methods.

*Significantly different from *CLV3::(CLV3)$_5$; CLV3::GUS* (P<0.001; Student's *t*-test).

†Significantly different from *CLV3::GUS* (P<0.01, Student's *t*-test).

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*

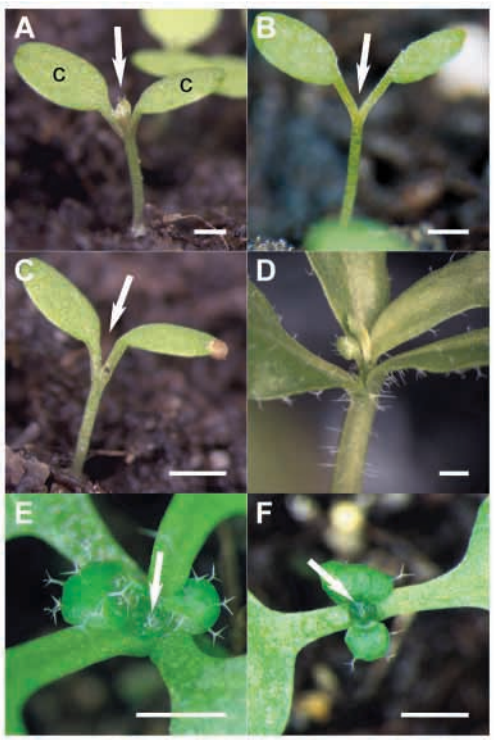

Fig. 5. Action of the *CLV* pathway on the *WUS* promoter. (A-F) Light micrographs of live plants. (A) Wild-type seedling 1 week after germination. The first true leaves (arrow) are visible between the cotyledons (c). (B,C) *wus-1* mutant (B) and *CLV1::CLV3*-expressing (C) seedlings one week after germination. No leaves are visible between the cotyledons (arrow), indicating a defective SAM. (D) Terminated inflorescence of a *CLV1::CLV3*-expressing plant. (E,F) *CLV1::WUS* (E)- and *CLV1::WUS; CLV1::CLV3* (F)-expressing seedlings 12 days after germination. In both cases, the meristem is considerably enlarged (arrow) compared to wild type. Scale bars: 1 mm.

mutants: they lacked a functional seedling meristem and repeatedly formed adventitious meristems, which terminated prematurely, and later flowers lacking the central gynoecium (Fig. 5A-D). This suggests that *CLV1::CLV3* expression produced a fully penetrant phenocopy of the *wus* mutant.

By contrast, *CLV1::CLV3*; *CLV1::WUS* coexpressing seedlings (n=28), in which the presence of the *CLV3* transgene was confirmed by PCR (data not shown), were indistinguishable from seedlings expressing only *CLV1::WUS* (Schoof et al., 2000), with a strongly enlarged shoot meristem (Fig. 5E,F). This indicates that the *WUS* cDNA expressed from the heterologous *CLV1* promoter in this experiment is not affected by ectopic *CLV3* expression.

Thus, *CLV* signalling probably acts by repressing transcription from the *WUS* promoter.

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 distribution of the CLV3-GFP fusion qualitatively reflects that of 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).

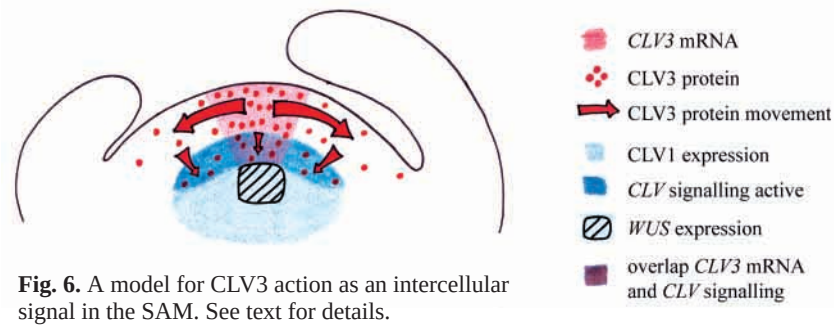

Fig. 6. A model for CLV3 action as an intercellular signal in the SAM. See text for details.

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)*$_5$-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)*$_5$-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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