The CLAVATA3/ESR Motif of CLAVATA3 Is Functionally Independent from the Nonconserved Flanking Sequences[1]

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It is believed that *CLAVATA3* (*CLV3*) encodes a peptide ligand that interacts with the CLV1/CLV2 receptor complex to limit the number of stem cells in the shoot apical meristem of *Arabidopsis thaliana*; however, the exact composition of the functional CLV3 product remains a mystery. A recent study on *CLV3* shows that the CLV3/ESR (CLE) motif, together with the adjacent C-terminal sequence, is sufficient to execute *CLV3* function when fused behind an N-terminal sequence of ERECTA. Here we show that most of the sequences flanking the CLE motif of CLV3 can be deleted without affecting CLV3 function. Using a liquid culture assay, we demonstrate that CLV3p, a synthetic peptide corresponding to the CLE motif of CLV3, is able to restrict the size of the shoot apical meristem in *clv3* seedlings but not in *clv1* seedlings. In accordance with this decrease in meristem size, application of CLV3p to in vitro-grown *clv3* seedlings restricts the expression of the stem cell-promoting transcription factor *WUSCHEL*. Thus, we propose that the CLE motif is the functional region of CLV3 and that this region acts independently of its adjacent sequences.

Stem cells positioned in the central zone of the plant shoot apical meristem (SAM) are the source of totipotent cells, which continuously give rise postembryonically to new organs (Steeves and Sussex, 1989; Weigel and Jürgens, 2002). These slow-dividing cells simultaneously maintain two antagonistic events, cell proliferation and cell differentiation, in a manner similar to animal stem cells (Groß-Hardt and Laux, 2003; Ohlstein et al., 2004; Scheres, 2005). Determination of the fate of the meristem progeny cells occurs by a population-based mechanism in which signals from neighboring cells play the most important role (Spradling et al., 2001; Weigel and Jürgens, 2002). Genetic experiments have shown that, as part of a feedback regulatory loop, the stem cell-promoting homeodomain transcription factor *WUSCHEL* (*WUS*), which is expressed in the stem cell organizing center (OC), provides a positive signal to maintain an undifferentiated state, whereas CLAVATA3 (CLV3) interacts with the underlying CLV1/CLV2 receptor complex to generate a negative signal that limits *WUS* expression and, in this way, is able to restrict the number of stem cells (Brand et al., 2000; Schoof et al., 2000). Thus, mutation in *WUS* leads to a termination of the SAM, whereas mutations in any of the *CLV* genes results in the expansion of *WUS* expression and subsequently an enlarged SAM with an increased number of stem cells (Clark et al.,1997; Mayer et al., 1998; Fletcher et al., 1999; Jeong et al., 1999). *CLV3* is able to restrict its own expression by preventing the differentiation of the cells in the central zone (Reddy and Meyerowitz, 2005).

Genetic data suggest that *CLV3* encodes a mobile ligand that acts in a non-cell-autonomous fashion in intercellular communication (Trotochaud et al., 1999; Rojo et al., 2002; Lenhard and Laux, 2003). CLV3 belongs to a family of small proteins, named CLV3/ESR (CLE), found in plants and parasitic nematodes (Opsahl-Ferstad et al., 1997; Fletcher et al., 1999; Cock and McCormick, 2001; Hobe et al., 2003; Olsen and Skriver, 2003; Wang et al., 2005). CLE proteins share an N-terminal secretion signal (SS) and a conserved 14-amino acid CLE motif at or near their C termini. The internal sequence between the SS and the CLE motif

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is generally not conserved, and a C-terminal extension is only found in a few CLE members, including CLV3. Overexpression of several *CLE* genes, such as *CLV3*, *CLE19*, and *CLE40* from Arabidopsis (*Arabidopsis thaliana*) and *HgSYV46* from the nematode *Heterodera glycines*, causes a termination of root development (Casamitjana-Martinez et al., 2003; Hobe et al., 2003; Fiers et al., 2004; Wang et al., 2005; Strabala et al., 2006). In vitro application of synthetic peptides, CLV3p, CLE19p, or CLE40p corresponding to the CLE motif of their encoding CLE proteins, phenocopies the overexpression phenotype in a *CLV2*-dependent manner (Fiers et al., 2005). At the cellular level, these treatments lead to misspecified cell identity and premature differentiation of ground tissue daughter cells, suggesting that these CLE peptides are able to interact with or saturate an unknown cell identity-maintaining receptor complex in roots (Birnbaum et al., 2003; Fiers et al., 2005). Moreover, several *CLE* genes could complement *clv3* mutants when expressed under the control of *CLV3* regulatory elements, indicating functional conservation among different *CLE* members (Hobe et al., 2003; Wang et al., 2005; Ni and Clark, 2006). A recent study also showed that the CLE motif and its downstream sequence alone can complement the *clv3-1* mutation when fused behind the secretion machinery (an N-terminal SS fragment and downstream 46 amino acids) of ERECTA, demonstrating the critical importance of these regions (Ni and Clark, 2006). Whether the CLE motif of CLV3 itself is sufficient to perform CLV3 function remains to be examined.

In this study, several deletion constructs of *CLV3* were made and used to determine which domains are essential for CLV3 function by complementing the *clv3* mutant. Our results show that, besides the SS (Rojo et al., 2002), the only other essential domain is the CLE motif. Removal of the sequence between the SS and the CLE motif, or the sequence after the CLE motif, did not affect the complementation of the *clv3* mutant, whereas removal of the CLE box abolishes CLV3 function completely. Using an in vitro assay, we showed that the synthetic peptide CLV3p is able to restrict the size of the SAM in *clv3* seedlings, but not in *clv1*, by restricting *WUS* expression, suggesting that the peptide acts in a manner similar to the endogenous gene. Peptides from different CLE genes confer various degrees of complementation. As such, we provide evidence that the CLE motif of CLV3 and several other CLEs act independently of their flanking sequences.

RESULTS

Deletion Analysis of the *CLV3* Gene

Except for the CLE motif (Fig. 1, in frame), very little amino acid similarity could be identified among the various CLE proteins (Cock and McCormick, 2001). To examine whether these nonconserved sequences were critical for CLV3 function, several deletion constructs were made in which different domains of CLV3 were removed (Fig. 2A, constructs 2–5) and compared with the full-length *CLV3* gene (construct 1). In construct 2, the sequence between the SS and the CLE motif, including the two introns, was removed, whereas in construct 3 only the sequence after the second intron and the CLE motif was removed, leaving the two introns intact. In construct 4, all of the coding sequence downstream of the *CLE* motif was deleted, and, in construct 5, both the CLE motif and its downstream sequence were removed. These constructs, which were fused to the *CLV3* 5′ and 3′ regulatory elements (Hobe et al., 2003), were used to transform *clv3-2* to examine whether they could restore the phenotype to wild type. The *clv3-2* mutant has a typical cone-shaped SAM in which the leaf primordia appear only at the bottom half of the structure (Fig. 2E). Due to the enlargement of the floral meristem as well as the SAM, the number of carpels per silique was increased from two in the wild type to five in *clv3-2* (Clark et al., 1995). Carpel number was used as an indirect measure of meristem size (Clark et al., 1995; Ni and Clark, 2006). Restoration of the wild-type carpel number was observed in all transgenic plants carrying constructs with a CLE box (Fig. 2, F–M), whereas no restoration was observed in plants carrying a construct in which both the CLE domain and the C terminus were deleted (Fig. 2, N and O). Occasionally, some siliques in transgenic plants carrying construct 2 or 3 had three carpels, resulting in an average carpel number of slightly more than two (Fig. 2A; T_0 plants $n \geq 14$).

The size of SAM in transgenic plants was measured in median sections obtained through Nomarski optics

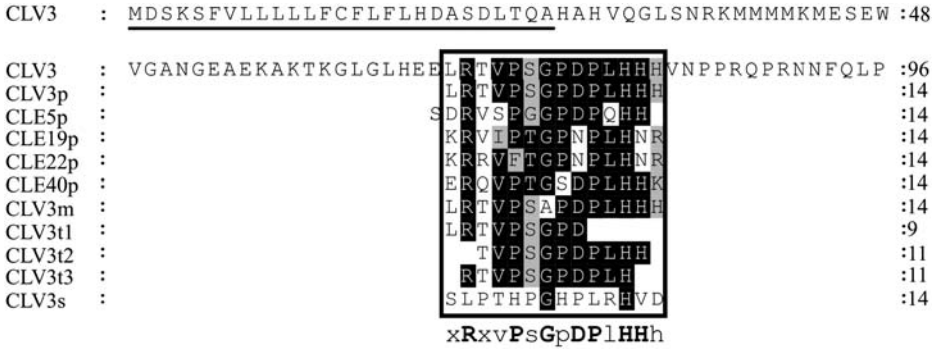

Figure 1. Alignment of CLV3 and the synthetic peptides used for the in vitro assay. Identical amino acids are shaded in black and similar ones in gray, the signal sequences are underlined, and the CLE box is framed with the consensus sequence underneath.

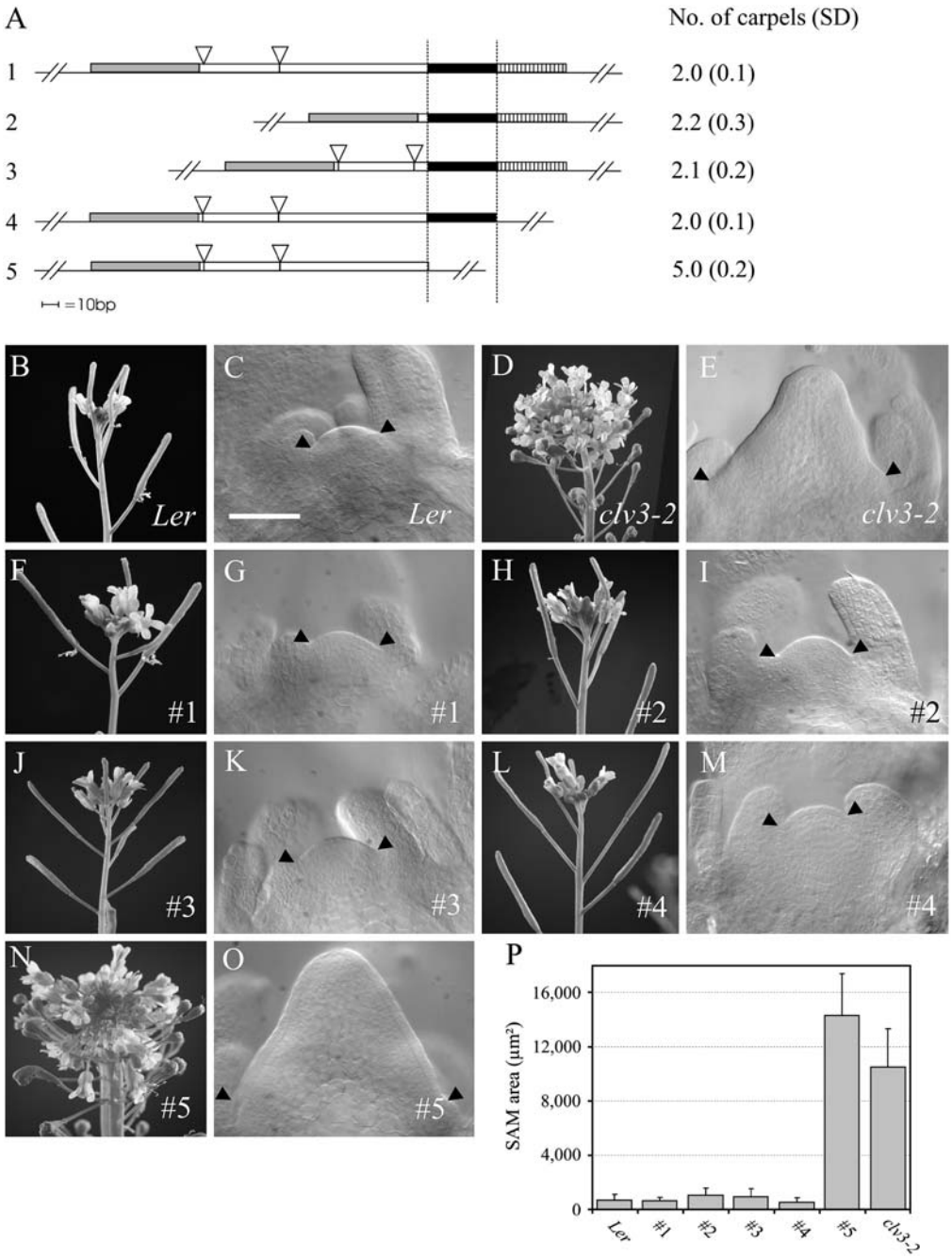

Figure 2. Functional analysis of *CLV3*. A, Full-length *CLV3* genomic sequence (construct 1) including 5′ upstream and 3′ downstream regulatory sequences (line with breaks) and four deletion constructs (constructs 2–5) were made by removing the sequence between the SS and the CLE motif (construct 2), part of this sequence (construct 3), the sequence after the CLE motif (construct 4), or the CLE motif and the sequence after (construct 5). The SS is shown in gray, the CLE motif in black, the sequence between the SS and the CLE motifs in white, and the sequence after the CLE motif with vertical lines. The positions of two introns are depicted with arrowheads. Data and error bars on the right represent the mean number of carpels per silique from transgenic *clv3-2* (for each construct, minimal 14 T_0-transgenic plants with 30 siliques per plant were counted). B to E, Inflorescence and meristem of wild type (L*er*), Arabidopsis (B and C), and *clv3-2* (D and E). F to O, Inflorescence and meristem of transgenic *clv3-2* carrying constructs 1 (F and G), 2 (H and I), 3 (J and K), 4 (L and M), and 5 (N and O), respectively. Note the restoration to wild type in transgenic *clv3-2* plants carrying constructs 1 to 4. The bar in C represents 50 μm for all SAM images. Arrowheads indicate the lower edges of the SAM; the area of the SAM was measured above a straight line between these two arrowheads. P, Area of the SAM from *clv3-2*, wild type, and different transgenic lines. The data and error bars represent the mean area in median sections $\pm$SD (T_1, $n \geq 14$).

(Fig. 2, C, E, G, I, K, M, and O). The results showed that all constructs containing the CLE domain restored the SAM more or less to the size of the wild type (692 ± 454 μm^2 for Landsberg *erecta* [L*er*]), ranging from 550 ± 330 μm^2 for plants carrying construct 4 to 989 ± 495 μm^2 for construct 3. In contrast, plants carrying a construct without a CLE domain (construct 5) had a SAM size of over 14,233 ± 2,829 μm^2 (Fig. 2P), which is significantly different from transgenic plants carrying constructs 1 to 4 ($P > 0.01$). Why the SAM in these plants was slightly larger than in *clv3-2* (10,520 ± 2,812 μm^2) is not known. It is possible that the RNA or protein produced by construct 5 might affect the stability of endogenous CLEs, causing an enhanced phenotype. If this is the case, the effect must be specific for the SAM in the seedlings because the carpel number was not increased. No other phenotypes, such as the termination of the SAM, as seen in plants overexpressing *CLV3*, were observed in any of the transgenic lines.

In Vitro Peptide Assay

In a previous report, we showed that addition of CLV3p to solidified medium could not rescue the enlarged SAM phenotype of the *clv3-2* mutant (Fiers et al., 2005), suggesting that the peptide is either nonfunctional in the SAM or that it cannot be transported efficiently from the roots to the SAM. To ensure sufficient access of the peptide to the SAM, we developed a protocol in which seedlings were grown in a liquid culture to which peptides were added.

Besides CLV3p, five peptides derived from CLV3p were synthesized and used as controls (Fig. 1). They were (1) CLV3m, which is similar to CLV3p but with a single amino acid change from G to A, as in *clv3-1* and *clv3-5* mutants (Fletcher et al., 1999); (2) three truncated peptides of CLV3p in which certain amino acids were deleted from the ends of the peptides (CLV3t1–3); and (3) CLV3s, a peptide with the same amino acid composition as CLV3p but randomized in sequence. To examine sequence conservation among different CLEs, four additional peptides, CLE5p, CLE19p, CLE22p, and CLE40p, were made based on the CLE motif of CLE5, CLE19, CLE22, and CLE40, respectively. These four *CLE* members are all expressed in reproductive shoot apices (Hobe et al., 2003; Sharma et al., 2003; Fiers et al., 2004). All peptides were individually applied to liquid media in the same concentration (10 μM) as used in the previous root assay (Fiers et al., 2005).

The seedlings were examined at successive time points for changes in the size of the SAM. Seedlings of *clv3-2* treated with any of the CLV3p derivatives, CLV3m, CLV3s, CLV3t1, CLV3t2, or CLV3t3, displayed a large cone-shaped SAM, as seen in the control treatment without peptide (Fig. 3, A–C), whereas SAMs of seedlings treated with CLV3p were much smaller and resembled those of the wild type (Fig. 3, H and I). Consecutive samplings showed that the effect of CLV3p in restricting the SAM was most evident after 8 d of treatment (data not shown). No termination of the SAM was observed. The size of individual cells in the SAM was the same as in the nontreated seedlings, which excluded the possibility that the reduction in meristem size was caused by reduced cell expansion.

We digitally measured the surface area of the median section of cleared SAMs (Fig. 3L). Strikingly, the SAM of *clv3-2* treated with CLV3p was only about 1,096 ± 795 μm^2, which resembles that of L*er* seedlings but not that of *clv3-2*. None of the tested truncated peptides promoted a reduction in SAM size, demonstrating that sequence integrity of the CLE motif is required for its function. The specificity of CLV3p is further illustrated by the fact that CLV3m did not restore the *clv3-2* phenotype and further illustrates the concordance between the genetic mutation and in vitro data.

Similarly, peptides of CLE5p, CLE22p, CLE19p, and CLE40p were tested in liquid culture. The results showed that CLE40p could fully complement the *clv3-2* phenotype (Fig. 3G), whereas application of both CLE5p and CLE19p resulted in a partial reduction of around 50% in the SAM size (Fig. 3, D, F, and L). This is in agreement with previously reported complementation data (Hobe et al., 2003; Ni and Clark, 2006). However, CLE22p only led to a minor reduction on *clv3-2* SAM (Fig. 3, E and L). Statistical analysis showed that this reduction is not significantly different from the control without peptide ($P > 0.1$). This result differs from that obtained by a transgenic approach in which *PCLV3*:*CLE22* is able to partially complement *clv3-1* (Ni and Clark, 2006).

Although an arrest of SAM development was observed upon overexpression of the *CLV3* gene (Brand et al., 2000), a similar arrest was not observed after peptide treatment in liquid culture. This is different from the results obtained in roots, where both overexpression of *CLV3* and treatment with CLV3p led to a termination of the root meristem (Hobe et al., 2003; Fiers et al., 2005). One possibility could be the presence of a cuticle layer in the SAM, which may form a barrier for efficient peptide uptake.

To elucidate whether CLV3p acts through the CLV1/CLV2 complex in the same manner as endogenous CLV3, we incubated the *clv1-1* mutant with CLV3p, CLV3m, CLV3s, and CLE40p. No significant differences were observed in the SAM (Fig. 3, J, K, and M), suggesting that a functional CLV1 receptor is required for the perception of the peptide signal.

Stability of the Peptide

Our data above showed that treatments with CLV3p or CLE40p did not lead to termination of the SAM, a result that differs from those obtained by overexpressing *CLV3* or *CLE40* (Brand et al., 2000; Hobe et al., 2003). Furthermore, although the size of the *clv3-2* SAM reverted almost to that of the wild type (L*er*) after 8 d of treatment, it expanded again after a prolonged incubation (data not shown). Therefore, we speculated that these peptides may have been degraded during incubation. To test this, we examined the peptide stability

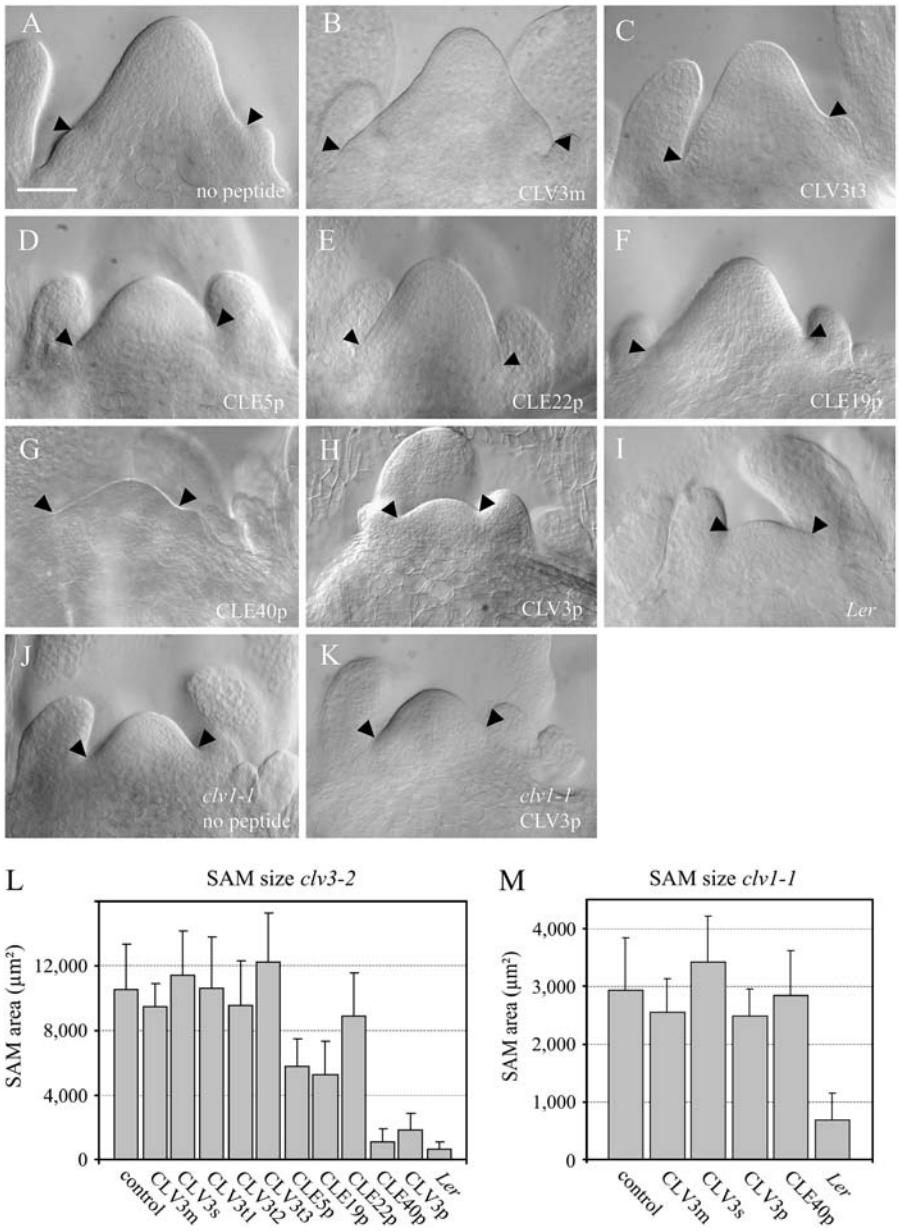

Figure 3. Effect of different peptides on the SAMs of *clv3-2*. A to H, SAM of *clv3-2* seedlings incubated with no peptide (A) or 10 μM CLV3m (B), CLVt3 (C), CLE5p (D), CLE22p (E), CLE19p (F), CLE40p (G), and CLV3p (H) for 8 d and examined with Nomarski optics. Note a nearly full restoration of the size of the SAM to wild type upon incubation with CLE40p (G) or CLV3p (H). I, SAM of an 8-d-old L*er* seedling. J and K, SAM of *clv1-1* seedlings incubated with no peptide (J) or CLV3p (K). L, Area of the SAM of *clv3-2* after different peptide treatments for 8 d in comparison with L*er*. M, Area of the SAM of *clv1-1* after peptide treatments for 8 d in comparison with L*er*. The area of the SAM was measured on a median plane by calculating the area above the straight line from the upper edges of two opposite leaf primordia (arrowheads). The data and error bars represent the mean ± SD ($n \geq 20$ in a minimum of two independent experiments). The bar in A represents 50 μm for A to K.

using matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). The results showed that CLV3p in medium without seedlings was rather stable, and no significant breakdown products were detected during a culture period of 2 weeks (data not shown). In contrast, the first sign of peptide degradation was observed in peptide-containing media incubated with seedlings after 2 d (Fig. 4A), after which CLV3p was gradually degraded and was no longer present on the eighth day of culture (Fig. 4, C and D). This seems to contradict the observation that the most evident effect of the peptides in restoring the *clv3-2* phenotype was observed on the eighth day of treatment. We believe that the delayed response reflects the time course required for the signal to be incorporated into the developmental program of the SAM.

One of the major truncated degradation products of the CLE peptides had a molecular mass of 1,156 D and was consistently found during incubation. MALDI-TOF analysis of the fragment showed that one amino acid was removed from the N terminus and two from the C terminus (Fig. 4C), and this truncated peptide therefore corresponds to the synthesized CLV3t3 used in this study. Further experiments with CLV3t3 showed that it was unable to restrict the size of the SAM in vitro, suggesting that this degradation might be nonspecific. The rapid degradation of the peptides in the media may partially explain why (1) the size of the SAM of *clv3* enlarges gradually after 8 d, (2) a relatively high concentration of peptide is needed for complementation in the in vitro culture experiments, and (3) no termination of the SAM was observed.

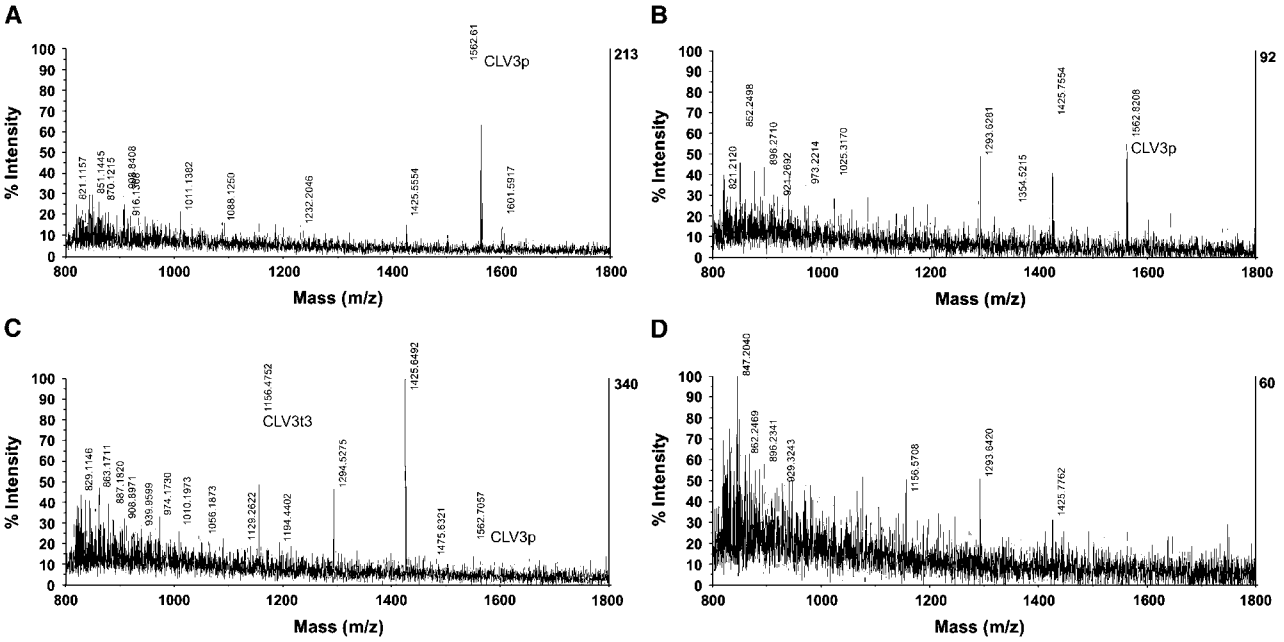

Figure 4. CLV3p is gradually degraded during incubation with Arabidopsis seedlings. A to D, MALDI-TOF MS analysis of the media during the course of incubation with seedlings of Arabidopsis (L*er*) after 2 d (A), 5 d (B), 7 d (C), and 8 d (D). CLV3p and CLV3t3 peaks are indicated.

WUS Expression Pattern Is Restored upon CLV3p Peptide Treatment

We addressed whether CLV3p, like the full-length CLV3 protein, acts by restricting the size of SAM via the WUS transcription factor (Brand et al., 2000; Schoof et al., 2000). In situ hybridization was performed to examine *WUS* expression in the *clv3-2* mutant after peptide treatments. *WUS* transcripts in *clv3-2* were observed in a large span of cells in the upper L3 and occasionally in the L2 layers of the SAM but excluded from the pith region (Fig. 5A), which is consistent with results observed previously (Brand et al., 2000; Schoof et al., 2000). After treatment with CLV3m, the *WUS* expression pattern was almost identical to that of the nontreated seedlings (Fig. 5, A and B). In contrast, seedlings treated with CLV3p for 8 d had a much smaller and condensed *WUS* domain (Fig. 5C), as seen in wild-type seedlings (Mayer et al., 1998; Schoof et al., 2000), and the number of cells expressing *WUS* was also reduced significantly (Fig. 5, A and C).

DISCUSSION

CLV3 is believed to function as a mobile ligand that binds to the CLV1/CLV2 receptor complex (Fletcher et al., 1999; Brand et al., 2000; Schoof et al., 2000). Although the SS of CLV3 has been shown to be functional, it has been proven to be difficult to detect the presence of the endogenous CLV3 product (Rojo et al., 2002; Fiers et al., 2004). The only endogenous CLE protein that has been detected in plants is the ESR protein in maize (*Zea mays*; Bonello et al., 2002). A recent report using domain swap experiments and complementation analysis suggested that the CLE motif is of critical importance for CLV3 function (Ni and Clark, 2006). In this article, we provide direct evidence to show that all sequences, except the SS and the CLE motifs, can be removed without affecting the CLV3 function. Furthermore, treatment of seedlings in a liquid culture with peptides corresponding to the

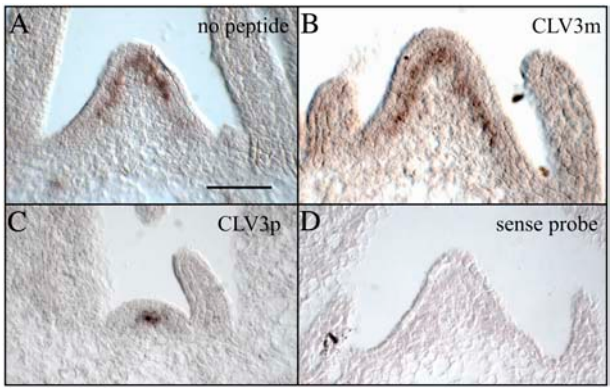

Figure 5. *WUS* expression in *clv3-2* upon incubation with CLV3p. A to C, In situ hybridization using an antisense probe of *WUS* cDNA showing the *WUS* expression in the SAM of *clv3-2* after 8 d of treatment. Treatment with no peptide (A) or CLV3m (B) resulted in an expanded *WUS* expression as compared to *clv3-2* treated with CLV3p (C) in which the *WUS* expression is restricted to the OC as seen in wild type. D, Control sample treated with CLV3m and hybridized with the *WUS* sense probe. The bar in A represents 50 μm for all samples.

CLE motif of CLV3 and several other CLEs restricted the size of the SAM via a *CLV1*-depedent pathway.

The CLE Motif and the SS of CLV3 Are Sufficient to Complement *clv3*

Deletion analysis showed that only the SS and the CLE motif are necessary for CLV3 function, which allows us to exclude the possibility that the flanking sequences contribute to the function of CLV3. This is in agreement with the results from domain swap experiments in which the SS and the variable sequence between the SS and the CLE motif could be replaced by a similar fragment from ERECTA (Ni and Clark, 2006). Whether processing is required to generate a CLV3 ligand and how such processing might take place and the nature of the recognition site remain to be investigated. Research in this direction is of general interest because several peptide ligands, such as phytosulfokine and systemin, have been identified biochemically in plants (Pearce et al., 1991; Yang et al., 1999), but no cleavage recognition sites have been found yet.

CLV3p Is Functional in Vitro as a Short-Distance Diffusible Signal

Our previous work shows that synthetic peptides corresponding to the CLE motif of CLV3, CLE19, and CLE40, when applied to solid media with germinating seedlings, are able to mimic the overexpression phenotype of these three genes, leading to a termination of root meristem development (Hobe et al., 2003; Fiers et al., 2004, 2005). Here, using a liquid culture system, we demonstrate that the 14-amino acid CLV3p can complement the meristem phenotype of *clv3-2* seedlings, restoring the size of the SAM to that of wild type. The extracellularly supplied 14-amino acid peptide (CLE motif of CLV3) is therefore necessary and sufficient for CLV3 function (Fig. 3H). The reversion of the SAM after peptide treatment suggests a reversible interaction of CLV3p with its receptors. Considering our earlier experiments in which no complementation was observed in the SAM when the peptide was applied to the roots in solid medium, we believe that the peptide may act as a short-distance diffusible signal that moves through the intercellular spaces, as was observed in the root tip (Fiers et al., 2005).

Different CLE Genes May Act in a Similar Manner

Examination of peptides corresponding to several other CLEs in the in vitro culture assay revealed a high functional conservation among different members of the CLE family. The differences between the *CLE* genes in complementing the *clv3* phenotype were also observed using a genetic approach (Ni and Clark, 2006). A major inconsistency between the peptide assay and the transgenic analysis is for *CLE22*, which is partially functional in complementing *clv3-1* in the transgenic approach (Ni and Clark, 2006) but was not able to restore the *clv3-2* mutation in the in vitro peptide assay. Two reasons could explain this discrepancy. First, it may result from the difference in alleles used in each study; *clv3-1* (with a point mutation in the CLE motif and an intermediate phenotype) used by Ni and Clark (2006) is weaker than *clv3-2* (a deletion in the coding region, resulting in a strong phenotype) used in this study. Second, the differences in developmental stages may also contribute to the differences. The peptide assay was performed at the seedling stage and was quantified using the size of the primary meristem, whereas the transgenic assay is based on the phenotype at the mature stage (carpel numbers in the fruits; Ni and Clark, 2006). It is possible that the unknown receptor involved in perception of CLE22, as pointed out by Ni and Clark (2006), is not available at the seedling stage.

The observations that not all *CLE* genes are interchangeable and that they might therefore interact with different receptors point out an additional complexity in the signal perception of the CLE proteins. It helps to explain why transgenic plants overexpressing *CLE19* and several other *CLE* genes do not show termination of the SAM as observed upon overexpression of *CLV3*, *CLE40*, or even *HgSYV46* (Brand et al., 2000; Hobe et al., 2003; Fiers et al., 2004; Wang et al., 2005; Strabala et al., 2006).

CLV3p Is Unstable in Culture Medium

CLV3p applied to the culture medium of developing seedlings restricted the expansion of the SAM only for a limited period of time. This is in contrast to the overexpression of the *CLV3* gene under the control of the cauliflower mosaic virus 35S promoter, where a termination of the SAM was observed as a consequence of down-regulating *WUS* expression (Brand et al., 2000; Hobe et al., 2003). We believe that this difference can be explained by the observation that peptides applied to the medium were degraded in the presence of the seedlings in addition to the presence of a cuticle layer in the SAM, which may form a barrier for efficient peptide uptake.

One major processed product observed in the seedling assay was identical to CLV3t3, which removed two amino acids from the N terminus and one from the C terminus. Peptide assay showed that CLV3t3 could not complement *clv3*, suggesting that the processing may not be functionally relevant. Similarly, Ni and Clark (2006) have also observed N-terminal processing of CLV3 using a cauliflower extract, which cleaved heterologously produced glutathione *S*-transferase-CLV3 fusion protein on at least two positions, one after M41 and another one after R70 (identical to CLV3t2 at the N terminus). Because a glutathione *S*-transferase antibody was used to detect potential processing, any additional processing that occurred downstream of the CLE motif in combination with the observed cleavage may not be detected.

CLV3p Application in Vitro Mimics the Endogenous *CLV3* Gene

In the *clv3* mutant, the expression domain of the stem cell-promoting homeodomain transcription factor *WUS* is expanded to include a large number of cells in the L2 and L3 layers of the cone-shaped SAM, rather than a compact cell cluster in the L3 layer of wild-type SAMs (Brand et al., 2000). CLV3p applied in the culture medium appears to act in a fashion similar to the endogenous CLV3 protein because *WUS* expression was restricted to a small cluster of cells in the OC.

How the CLV3p peptide restricts the size of the SAM remains unknown. Most likely it triggers meristematic cells in the periphery into the differentiation and organogenesis program through a signal transmitted by the *CLV1/CLV2* receptor complex, after activation by CLV3, toward *WUS* (Brand et al., 2000; Schoof et al., 2000). To determine whether this is indeed the case, *clv1-1* seedlings were incubated with different CLE peptides. The *clv1-1* allele was selected because it carries a dominant-negative point mutation in the intracellular kinase domain of CLV1 that is thought to interfere with the function of both CLV1 and other overlapping receptor kinases (Clark et al., 1997; Diévart et al., 2003; DeYoung et al., 2006). The size of the SAM in *clv1-1* was not significantly changed upon incubation with CLV3p or any of the control peptides. Therefore, we propose that CLV3p, like endogenous CLV3, acts in a CLV1-dependent manner (Trotochaud et al., 1999), although we could not exclude the possibility that redundant receptor kinases are also involved.

In summary, our results showed that the CLE motif of CLV3 is sufficient to mimic CLV3 function both in vivo (together with the SS) and in vitro in a sequence-dependent manner. The data obtained so far support the hypothesis that the 14-amino acid CLE motif is the functional part of CLV3 in restricting the stem cell population in the SAM of Arabidopsis. The consistency between the in vitro peptide treatment and the in vivo transgenic approach with different *CLE* genes performed by Ni and Clark (2006) showed that the mode of action of the CLE peptides and the corresponding genes is the same and led us to propose that, in the case of CLV3, the CLE motif is the functional part and can act independently of the nonconserved flanking sequences.

MATERIALS AND METHODS

Peptide Assay

Wild type (L*er*), *clv1-1*, and *clv3-2* (all in a L*er* background) were provided by the Nottingham Arabidopsis Stock Centre (NASC). Seeds were gas sterilized in a desiccator for 1 h with a mixture of 100 mL of kitchen bleach (containing 4% sodium hypochlorite) and 3 mL of concentrated HCl. These seeds were incubated in liquid medium containing 0.5× Murashige and Skoog salt mixture (Duchefa), 1% Suc, 0.5 g/L MES, pH 5.8, for 3 d at 4°C. Afterward, the medium was refreshed and peptides were added at a concentration of 10 μM. Incubation was performed in 50-mL Falcon tubes (20 seeds/tube) with 6 mL of media on a roller bank, 23°C, 16 h light/day. All peptides were synthesized by MIMOTOPES with a purity of >80%.

Whole-Mount Examination

Shoot apices of 8-d-old seedlings were excised under a dissecting microscope, cleared following the protocol of Sabatini et al. (1999), and analyzed under a Nikon microscope equipped with Nomarski optics. From each SAM, a picture was taken from the median optical section. The surface area was defined by measuring the area of the meristem dome above a straight line between the top edges of the smallest leaf primordia. All areas were measured using the ImageJ program (http://rsb.info.nih.gov/ij).

MALDI-TOF MS Analysis

During incubation of the seedlings in peptide-containing media, samples were taken once a day and frozen immediately for later analysis. These samples were diluted 200 times with 0.1% trifluoric acid and spotted (0.5 μL each) on a stainless steel target, together with 0.5 μL of α-cyano-4-hydroxycinnamic acid (dissolved until saturation in 60% acetonitrile and 1% trifluoric acid) for analysis in a MALDI-TOF mass spectrometer (ABI4700; Proteomics Analyzer). As a control, the stability of CLV3p was measured by adding the peptide to the same medium without seedlings and incubating under the same conditions before analyzing as above.

In Situ Hybridization

Eight-day-old seedlings with or without peptide treatment were fixed for 2 h in a modified formaldehyde acetic acid solution (Liu et al., 1993) and embedded in paraffin. Sections with a thickness of 10 μm were prepared and used for nonradioactive in situ hybridization using the mRNA locater kit (Ambion), following the protocol of the manufacturer. A fragment of the *WUS* cDNA was amplified from Arabidopsis (*Arabidopsis thaliana*) cDNA, using two sets of primers (one with a T7 promoter), namely, 5′-ATATAATACGACTCACTATAGCTCGTGAGCGTCAGAAG-3′, 5′-GAAGCGTACGTCGATGTTC-3′ and 5′-ATATAATACGACTCACTATAGAAGCGTACGTCGATGTTC-3′, 5′-GCTCGTGAGCGTCAG AAG-3′. The first pair of primers was used to produce the sense and the latter pair was used to produce the antisense RNA probes through in vitro transcription using T7 polymerase labeled with digoxigenin (Roche). In situ hybridization and immunological detection were performed as previously described (Cañas et al., 1994).

Complementation Analysis

The full-length *CLV3* (construct 1) including the *CLV3* coding region (including two introns), plus a 1.8-kb promoter and 1.5-kb terminator (Hobe et al., 2003), was generated using the primers MF1 (5′-**GACAAGTTTGTACAAAAAAGCAGGCT**CAGTCTCTTGTCGCTTAACG-3′) and MF2 (5′-**GACCACTTTGTACAAGAAAGCTGGGT**GATCAATTACTAACTACAATGG-3′) including the Gateway cloning sites (in bold). The promoter and terminator fragments for the deletion constructs (constructs 2–5) were generated using MF1/MF3 (5′-ACAGTCCTTAACTCTTCATGAGAAGCATCATGAAGGAACA-3′) and MF2/MF4 (5′-TGTTCCTTCATGATGCTTCTCATGAAGAGTTAAGGACTGT-3′) for construct 2, MF1/MF5 (5′-ACAGTCCTTAACTCTTCATGCATCTGCCAATTGAACAAC-3′) and MF2/MF6 (5′-GTTGTTCAATTGGCAGATGCATGAAGAGTTAAGGACTGT-3′) for construct 3, MF1/MF7 (5′-AGCAACAAGAGATTAGGTCAATGATGGTGCAACGGGTCAG-3′) and MF2/MF8 (5′-CTGACCCGTTGCACCATCATTGACCTAATCTCTTGTTGCT-3′) for construct 4, and MF1/MF9 (5′-AGCAACAAGAGATTAGGTCACTCTTCATGTAGTCCTAAAC-3′) and MF2/MF10 (5′-GTTTAGGACTACATGAAGAGTGACCTAATCTCTTGTTGCT-3′) for construct 5, respectively. The promoter and terminator fragments were combined using PCR with overlapping primers, after which the complete deletion construct was generated via PCR using primers MF1 and MF2.

The PCR fragment was recombined into *pDONR207* (BP reaction) following the protocol of the supplier (Invitrogen). The binary vector was obtained with an LR reaction using the plasmids from the BP reaction mixed with the *pKGW* vector following the protocol of the supplier (Invitrogen). The binary vectors, containing the different *CLV3* fragments, were sequenced before transformation to *Agrobacterium tumefaciens* C58pmp90. The *clv3-2* plants were transformed using the floral-dip method as described (Clough and Bent, 1998).

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