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Contributions of Individual Amino Acid Residues to the Endogenous CLV3 Function in Shoot Apical Meristem Maintenance in *Arabidopsis*

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ABSTRACT As a peptide hormone, CLV3 restricts the stem cell number in shoot apical meristem (SAM) by interacting with CLV1/CLV2/CRN/RPK2 receptor complexes. To elucidate how the function of the CLV3 peptide in SAM maintenance is established at the amino acid (AA) level, alanine substitutions were performed by introducing point mutations to individual residues in the peptide-coding region of CLV3 and its flanking sequences. Constructs carrying such substitutions, expressed under the control of *CLV3* regulatory elements, were transformed to the *clv3-2* null mutant to evaluate their efficiencies in complementing its defects in SAMs *in vivo*. These studies showed that aspartate-8, histidine-11, glycine-6, proline-4, arginine-1, and proline-9, arranged in an order of importance, were critical, while threonine-2, valine-3, serine-5, and the previously assigned hydroxylation and arabinosylation residue proline-7 were trivial for the endogenous CLV3 function in SAM maintenance. In contrast, substitutions of flanking residues did not impose much damage on CLV3. Complementation of different alanine-substituted constructs was confirmed by measurements of the sizes of SAMs and the *WUS* expression levels in transgenic plants. These studies established a complete contribution map of individual residues in the peptide-coding region of CLV3 for its function in SAM, which may help to understand peptide hormones in general.

Key words: CLV3; peptide; stem cell maintenance; amino acid residue; contribution.

INTRODUCTION

Peptides have long been recognized as hormones in animals, which act as intercellular communication signals in endocrinal and neuronal systems. Usually, peptides are produced as preproproteins, which are cleaved and sometimes modified to generate small functional peptides (Hook et al., 2008). Since the first plant peptide hormone, systemin, was discovered two decades ago (Pearce et al., 1991), many small peptides have been identified, which play crucial roles in plant development and defense responses (for recent reviews, see Matsubayashi and Sakagami, 2006; Boller and Felix, 2009; Katsir et al., 2011). Compared to traditional phytohormones, peptides in theory have more flexibility in changing their primary structures and subsequently their specificities in evolution through AA substitutions.

CLAVATA3 (CLV3) acts as a small peptide to interact with its putative receptors including CLAVATA1 (CLV1), CLAVATA2 (CLV2), SUPPRESSOR Of LLP1 2 (SOL2)/CORYNE (CRN), and RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) to repress the expression of a stem cell-promoting homeodomain transcription factor *WUSCHEL* (*WUS*) in SAMs (Clark et al., 1993; Kayes and Clark, 1998; Fletcher et al., 1999; Brand et al., 2000; Schoof et al., 2000; Miwa et al., 2008; Müller et al., 2008; Kinoshita et al., 2010). Mutations of *CLV1*, *CLV2*, and *CLV3* result in expanded SAMs and increased numbers of floral organs (Clark et al., 1995; Fletcher et al., 1999). *CLV1* encodes a leucine-rich repeat (LRR) receptor kinase (Clark et al., 1997), *CLV2* encodes a LRR receptor-like protein lacking the kinase domain (Jeong et al., 1999), and *SOL2/CRN* encodes a receptor-like kinase with a short extracellular domain (Miwa et al., 2008; Müller et al., 2008). Biochemical studies show that CLV3 binds to the ectodomain of CLV1 (Kondo et al., 2008; Ogawa et al., 2008). A current working model proposes that the CLV3 peptide is primarily perceived either by a multimeric receptor complex comprising CLV1, CLV2, and CRN, or two parallel complexes, one with CLV1 homodimer and one with CLV2 and CRN heterodimer (Bleckmann et al., 2010; Guo et al., 2010; Zhu et al., 2010). Another LRR receptor-like kinase, RPK2, is found to

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sense the CLV3 signal in a homodimer, and to act in parallel with CLV1/CLV2/CRN receptor kinases (Kinoshita et al., 2010). These studies suggest that complicated perception machinery is involved in the CLV3 signal transduction.

CLV3 encodes a 96-AA precursor that belongs to the CLV3/ESR (CLE) family of proteins sharing a conserved 14-AA CLE motif at or near their C-terminals (Cock and McCormick, 2001). In *Arabidopsis* genome, there are at least 83 CLE members that share a conserved CLE motif, with the potential to produce 32 different CLE peptides (Oelkers et al., 2008). Different *CLE* genes have been implicated in regulating meristem development, xylem differentiation, cell proliferation, and nematode–plant interactions (Fletcher et al., 1999; Fiers et al., 2005; Wang et al., 2005; Ito et al., 2006; Hirakawa et al., 2010). When overexpressed in *Arabidopsis*, 19 out of 26 investigated CLE peptides are able to trigger the consumption of root meristems (RMs), and 10 generate a common shrub-like phenotype (Fiers et al., 2004; Strabala et al., 2006; Kinoshita et al., 2007). These results suggest that functional redundancies are present among CLE members, while, in the endogenous situation, the specificity may be achieved by tissue-specific expressions (Jun et al., 2010). Such a functional redundancy has also been observed in other peptide-coding genes and between different peptide families (Wen et al., 2004; Lee et al., 2011).

Using domain deletion analyses and *in vitro* peptide assays, it has been shown that, in addition to the N-terminal signal peptide, the CLE motif of CLV3 is essential and sufficient to complement *clv3-2* defects (Fiers et al., 2006). Synthetic peptides corresponding to the 14-AA CLE motifs of CLV3, CLE19, or CLE40 are functional *in vitro* in triggering premature differentiation of stem cells in RMs (Fiers et al., 2005). Through the use of in-situ MALDI–TOF MS analyses, the endogenously functional CLV3 is first identified to be a 12-AA hydroxylated peptide derived from the CLE motif (Kondo et al., 2006). Later, a 13-AA hydroxylated and arabinosylated peptide is found by the nano-LC–MS/MS analysis in transgenic plants overexpressing *CLV3*, and the modification on the second proline enhances the activity of the CLV3 peptide (Ohyama et al., 2009). *In vitro* peptide assays performed in roots using alanine-substituted CLV3 peptides indicate that individual residues of the CLE motif contribute differently to the termination of RMs (Kondo et al., 2008). However, since *CLV3* is not expressed in roots, these responses most likely represent the interactions with non-specific receptors such as CLV2, CRN, and RPK2 available in roots (Fiers et al., 2005; Müller et al., 2008; Kinoshita et al., 2010).

The availability of a null mutant *clv3-2* with strong phenotypes provides an opportunity to perform *in vivo* complementation to examine contributions of individual residues in CLV3. In this study, we generated a series of constructs carrying the complete regulatory and coding regions of *CLV3* with alanine-substitutions of every AA residue in the 12-AA CLE motif (residues arranged in an order of R1, T2, V3, P4, S5, G6, P7, D8, P9, L10, H11, and H12; named the 'core CLE motif' hereafter; Figure 1) and its flanking sequences (residues H-3, E-2, E-1, and L0 at the left border, and H13, V14, N15, and P16 at the right border; Figure 1). These constructs were transformed one by one to *clv3-2* to complement the defects in meristem sizes and floral organ number. We found that residues D8, H11, G6, P4, R1, and P9 were important for the CLV3 function in SAMs. However, substitutions of residues in the flanking sequences and the previously assigned modification residue P7 still achieved almost complete complementation in *clv3-2*, suggesting these residues were trivial to the CLV3 function. We further showed that the complementation efficiencies of different alanine-substituted constructs were confirmed by examination of the sizes of SAMs and the *WUS* expression levels in transgenic plants. These studies established a complete contribution map of every residue in the peptide-coding region of the CLV3 for its function in SAMs.

RESULTS

In vivo Analysis of Residues Critical for CLV3 in Restricting the Floral Organ Numbers

To elucidate the contribution of individual AA residues in the core CLE motif of CLV3 (Figure 1) in the SAM, *in vivo* complementation analyses were performed to deliver alanine-substituted

Figure 1. Constructs for *In vivo* Complementation Analyses.

Point mutations were introduced to the peptide-coding region and its flanking sequences of *CLV3* to substitute individual AA by alanine. *CLV3F*, a construct carrying the 3,934-bp wild-type full-length *CLV3* genomic sequence including the 5′ and 3′ regulatory elements and coding regions; *R1A* to *P16A*, 21 constructs carrying point mutations that substitute individual AA in the core CLE motif and its flanking sequences, one at a time, to alanine through a PCR-based mutagenesis; *L0A_H13A*, a construct carrying double point mutations on two junction residues L and H; black box, exon; arrows, 5′ and 3′ regulatory elements; line, intron; letter in red, substituted residues; gray-shaded box, the core CLE motif.

CLV3 genes to *clv3-2*. A PCR-based method was used to create alanine substitutions in the core CLE motif of CLV3, one at a time (Figure 1). The 3,934-bp full-length *CLV3* genomic DNA, including regulatory elements (a 1857-bp 5′ upstream region and a 1516-bp 3′ downstream region; Brand et al., 2002) was used to ensure proper expression of the transgene. Twelve constructs (designated *R1A*, *T2A*, *V3A*, *P4A*, *S5A*, *G6A*, *P7A*, *D8A*, *P9A*, *L10A*, *H11A*, and *H12A*) were made and transformed into *clv3-2* (Fletcher et al., 1999) by using the floral dip method (Clough and Bent, 1998). A construct carrying the native *CLV3* genomic sequence (named *CLV3F*) was used as a control. For every construct, at least 25 independent T1 transgenic plants were obtained, and the carpel number was counted to quantify the complementation efficiency, as described before (Ni and Clark, 2006). For each plant, six siliques located at the bottom, middle, and top of the primary inflorescences were examined for their carpel number. The silique in the wild-type (L*er*) typically comprises two carpels, while, in *clv3-2*, it usually has five to seven (Leyser and Furner, 1992; Fletcher et al., 1999). Our analyses showed that each residue in the core CLE motif tolerated alanine substitutions differently (Figure 2A). In the control, all T1 plants carrying *CLV3F* showed two-carpel phenotypes (Figure 2A), which indicated an effective complementation of *clv3-2* defects by the transgene. By contrast, less than 25% of plants carrying the *D8A*, *H11A*, *G6A*, *P4A*, *R1A*, or *P9A* construct exhibited the two-carpel phenotype (Figure 2A), suggesting that residues D8, H11, G6, P4, R1, and P9, arranged in an order of importance, are critical for CLV3. More than 42% of transgenic *clv3-2* plants carrying *T2A*, *V3A*, *S5A*, *P7A*, *L10A*, or *H12A* showed the wild-type two-carpel phenotype in siliques (Figure 2A), indicating these residues are less important for CLV3.

Unlike the usual four-whorl flower in the wild-type, *clv3-2* has an extra fifth whorl of ovaries in the silique (Fletcher et al., 1999). To determine to what extent the alanine-substituted *CLV3* transgenes complement such a defect, we examined the presence of the fifth whorl ovaries in transgenic plants. Different alanine-substituted constructs showed variable efficiencies in complementing the fifth-whorl ovary phenotype (Figure 2B). These ovaries disappeared after being transformed with *CLV3F*, *T2A*, *V3A*, or *S5A* (Figure 2B). However, plants carrying the *D8A* construct failed to complement such a defect completely (Figure 2B), which confirmed the critical importance of the D8 residue in CLV3 (Figure 2B). These observations are consistent with data obtained from counting the carpel numbers, indicating that the same signaling pathway was involved in these two whorls.

Decreased Floral Organ Number in Transgenic *clv3-2* Correlated with Restricted Sizes of SAMs

To verify whether different alanine-substituted constructs indeed gave different degrees of complementation of *clv3-2*

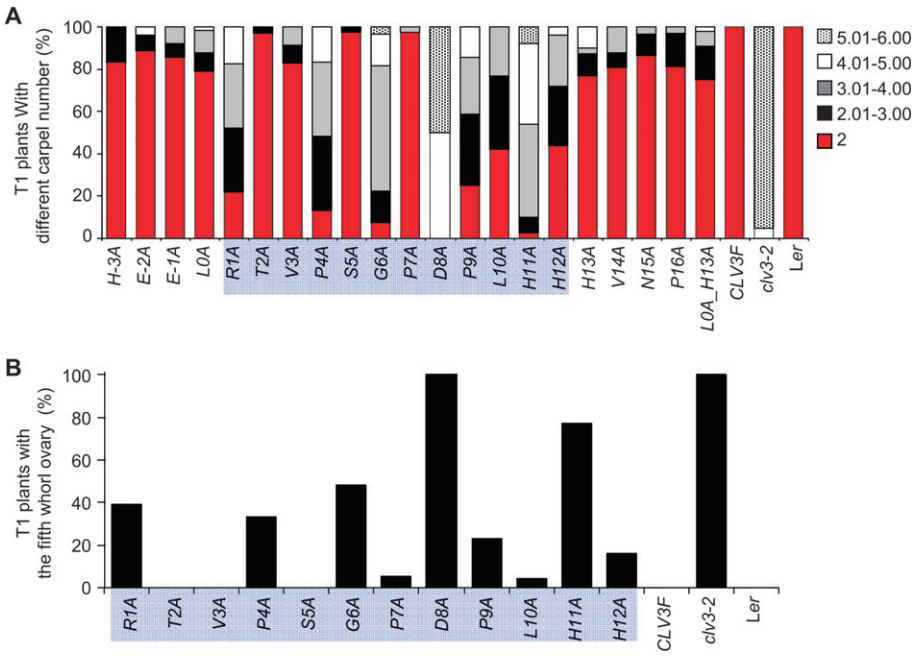

Figure 2. Complementation Efficiencies of Different Alanine-Substituted Constructs in Transgenic *clv3-2* Lines.

(A) Contributions of individual AAs in the core CLE motif and its flanking AA residues to CLV3 functions *in vivo*, measured by percentage of T1 transgenic *clv3-2* lines with different carpel numbers. Plants of *clv3-2*, L*er*, and transgenic *clv3-2* carrying full-length *CLV3* (*CLV3F*) were used as controls. Percentages of T1 lines with two carpels (a complete complementation) are shown in red.

(B) Contributions of AA residues in the core CLE motif in complementing the fifth whorl ovary phenotype of *clv3-2*, measured by percentage of T1 transgenic *clv3-2* lines with the fifth whorl ovary.

Shaded in light blue, the core CLE motif.

defects in SAMs, we examined SAMs in 9-day-old transgenic T2 seedlings carrying *R1A*, *T2A*, *V3A*, *P4A*, *S5A*, *G6A*, *P7A*, *D8A*, *P9A*, *L10A*, *H11A*, or *H12A* under a differential interference contrast (DIC) microscope, and analyzed with ImageJ, as described previously (Fiers et al., 2005). The average size of SAMs in *clv3-2* was 7576 ± 304 μm^2 (Figure 3A and 3B). While the average size of SAMs in *clv3-2* carrying *CLV3F* was 1043 ± 303 μm^2 (Figure 3A and 3B), which was quite close to that in L*er* (673 ± 387 μm^2; Figure 3A and 3B). The sizes of SAMs in transgenic *clv3-2* carrying *D8A* or *G6A* were about half of those in *clv3-2* (Figure 3A and 3B), suggesting the critical importance of the D8 and G6 residues. Similarly, the SAM sizes in transgenic *clv3-2* carrying *R1A*, *P9A*, *P4A*, and *H11A* were 1942 ± 707, 1859 ± 353, 1606 ± 621, and 1270 ± 600 μm^2, respectively (Figure 3A and 3B), illustrating the relatively strong contributions of these residues to CLV3. However, sizes of SAMs in transgenic plants carrying *T2A*, *V3A*, *S5A*, *P7A*, *L10A*, or *H12A* constructs were not significantly different from those carrying *CLV3F* (Figure 3A and 3B), confirming that residues T2, V3, S5, P7, L10, and H12 are less important for CLV3.

Residues Flanking the Core CLE Motif Contribute Very Little to the CLV3 Function

To address to what extent the flanking residues contribute to the CLV3 function, eight *CLV3* constructs (named *L0A*, *E-1A*, *E-2A*, *H-3A*, *H13A*, *V14A*, *N15A*, and *P16A*) with alanine-substitutions in four residues adjacent to the left border and four to the right border were made and transformed to *clv3-2* to examine their complementation efficiencies. We also made a construct with double substitutions (*L0A_H13A*) to replace two junction residues outside the core CLE motif at the same time (Figure 1). Results showed that 75–88% of the *clv3-2* plants transformed with these constructs gave complete complementation (Figure 2A), suggesting that none of these residues is critical for CLV3 (Figure 2A). It is evident that the flanking sequences outside of the core CLE motif contribute very little to the CLV3 function.

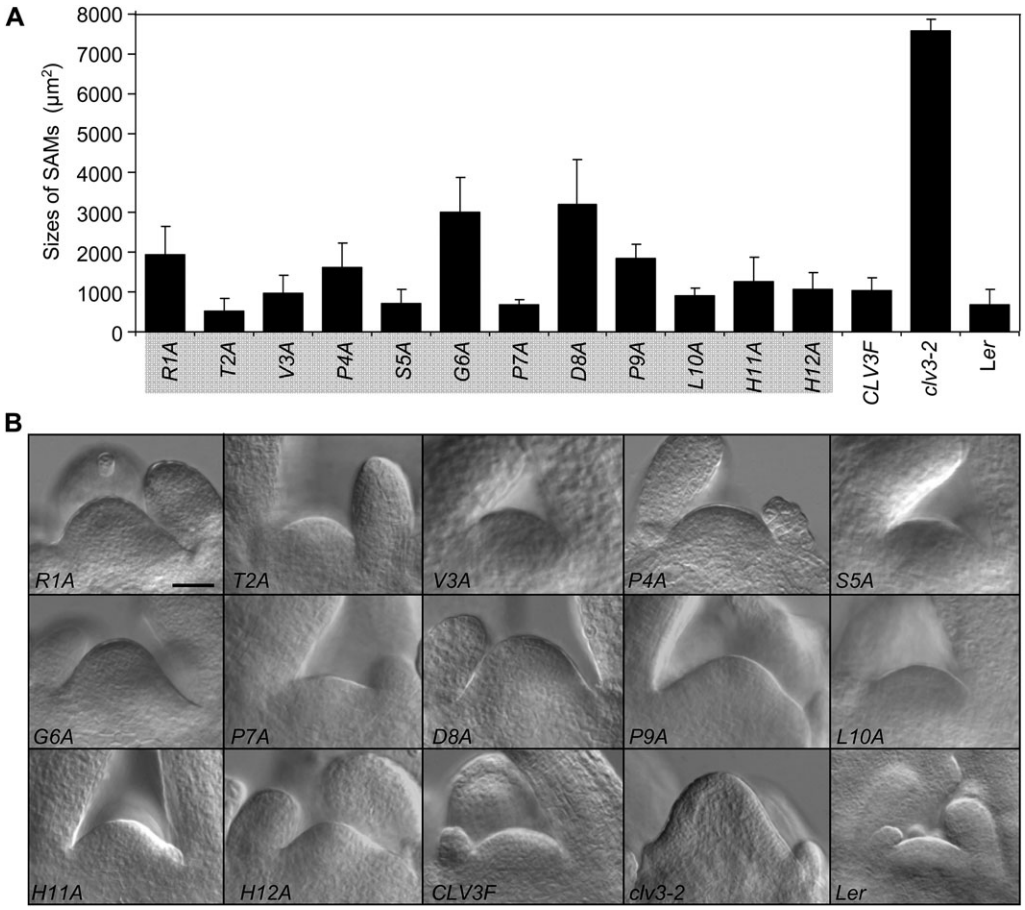

Figure 3. Sizes of SAMs in *clv3-2* Carrying Different Alanine-Substituted *CLV3* Constructs.

(A) SAM areas of T2 transgenic lines of *clv3-2* carrying alanine-substituted *CLV3* constructs.

(B) Morphologies of SAMs of T2 transgenic plants carrying *R1A*, *T2A*, *V3A*, *P4A*, *S5A*, *G6A*, *P7A*, *D8A*, *P9A*, *L10A*, *H11A*, or *H12A* constructs, as compared to controls of *CLV3F*, *clv3-2*, and L*er*. For each line, sizes of SAMs of at least 25 plants were measured and analyzed with the ImageJ software.Bar = 50 μm. *CLV3F*, *clv3-2* carrying a full-length *CLV3*; shaded, the core CLE motif.

WUS Expressions in Transgenic *clv3-2* Carrying Alanine-Substituted *CLV3* Constructs

CLV3 restricts the size of the SAM by repressing *WUS* expression (Brand et al., 2000; Schoof et al., 2000; Fiers et al., 2006). To address whether complementation efficiencies of alanine-substituted CLV3 in restricting the SAMs correlate with *WUS* expression levels, real-time PCRs were performed in RNA samples isolated from shoot apices of transgenic *clv3-2* T2 seedlings carrying *R1A*, *T2A*, *V3A*, *P4A*, *S5A*, *G6A*, *P7A*, *D8Ap*, *P9A*, *L10A*, *H11A*, and *H12A*. As shown in Figure 4, the *WUS* expression level in *clv3-2* was about 10-fold higher than that in the wild-type, whereas the *WUS* expression in *clv3-2* transformed with *CLV3F* was about the same as that in the wild-type (Figure 4). Similarly, levels of *WUS* expression in shoot apices of *clv3-2* transformed with *T2A*, *V3A*, or *P7A* were also about the same as that in the wild-type (Figure 4), indicating that these three substitutions have very little effect on the function of the *CLV3*. However, *clv3-2* transformed with *D8A* or *H11A* showed similar *WUS* expression levels as that in *clv3-2* (Figure 4), indicating that residues D8 and H11 are critical for the CLV3 functions. These studies suggested that differential complementation of alanine-substituted *CLV3* constructs in *clv3-2* correlate with their activities in suppressing *WUS* expressions.

DISCUSSION

Although *in vitro* assays have been used extensively to elucidate the functions of small peptides (Pearce et al., 1993; Matsubayashi et al., 1996; Bahyrycz et al., 2004; Pearce et al., 2008), to what extent results obtained from these assays correlate with endogenous peptide functions is not known. In this study, by taking advantage of the null mutant *clv3-2* and the easy transformation of *Arabidopsis*, we performed *in vivo* complementation analyses to examine the contributions of individual residues in CLV3 to its functions in stem cell maintenance in SAMs. Our studies yielded a complete contribution map for every residue in the core CLE motif and its flanking sequences of CLV3, which differs evidently from the specificity requirement obtained by using *in vitro* root assays (Kondo et al., 2008).

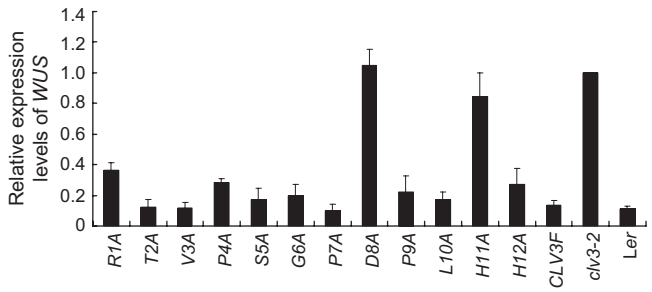

Figure 4. Real-Time PCR Analyses of *WUS* Expressions in Shoot Apices of *clv3-2* Transformed with Alanine-Substituted CLV3 Constructs.

Constructs used include *R1A*, *T2A*, *V3A*, *P4A*, *S5A*, *G6A*, *P7A*, *D8A*, *P9A*, *L10A*, *H11A*, and *H12A*. Plants carrying *D8A* or *H11A* showed the highest expression of *WUS*, compared to that in *clv3-2*. Plants carrying *T2A*, *V3A*, or *P7A* exhibited the lowest *WUS* expressions, similar to *clv3-2* transformed with *CLV3F*.

Through *in vitro* peptide assays in roots using alanine-substituted peptides, Kondo et al. (2008) evaluated the contribution of each residue in the core CLE motif of CLV3 for its function in triggering the termination of RMs, showing that residues R1, P9, H11, and H12, arranged in an order of importance, are critical, while T2, S5, P7, and L10 are the least important ones. Using *in vitro* competitive receptor binding assay, these authors also showed that residues R1, H12, G6, P4, and V3 are important, arranged in an order of importance, for binding to the CLV1 ectodomain. For the least important ones, the same four residues (T2, S5, P7, and L10) identified in the root assay are shown to be unimportant. These studies revealed some differences between *in vitro* peptide assays in roots and *in vitro* peptide–receptor binding assays (Kondo et al., 2008). This is not unexpected. In SAMs, known receptors involved in the perception of CLV3 peptides include CLV1, CLV2, SOL2/CRN, and RPK2 (Clark et al., 1993; Kayes and Clark, 1998; Miwa et al., 2008; Müller et al., 2008; Kinoshita et al., 2010), whereas receptors involved in the CLV3-triggered root meristem termination are CLV2, SOL2/CRN, and RPK2 (Fiers et al., 2005; Miwa et al., 2008; Müller et al., 2008; Kinoshita et al., 2010). Since *CLV3* is not expressed in root meristems, the activity of CLV3 peptide in RMs is most likely representing their non-specific interactions with some of these receptor proteins. To access contributions of individual residues of CLV3 in SAMs endogenously, we performed *in vivo* analyses through transforming the *clv3-2* with constructs in which residues in the core CLE motif and its flanking sequences were substituted by alanine, one at a time, and expressed under the control of native *CLV3* regulatory elements to examine their complementation efficiencies. These studies revealed that six residues (D8, H11, G6, P4, R1, and P9), arranged in an order of importance, are critical for CLV3 in restricting the size of SAMs (Figure 5).

We noticed that the R1 residue in the core CLE motif is consistently important for the CLV3 in all these assays. As a residue located at the junction, R1 might be implicated in peptide cleavage that is expected to occur right before it (Kondo et al., 2006; Ni and Clark, 2006; Ni et al., 2011). Another interesting fact is that the importance of H12 detected in two *in vitro* assays was not observed in our *in vivo* complementation analyses, suggesting an additional difference between *in vitro* and *in vivo* assays. There is no doubt that the *in vivo* complementation assay is the most accurate way to elucidate the function of CLV3, as stated by Ni et al. (2011). In our previous *in vivo* domain deletion experiments, it has been shown that removal of all 15 residues extended after residue H13 does not affect the function of CLV3 (Fiers et al., 2006). Most likely, different contributions in H12 between *in vivo* and *in vitro* assays are not caused by omission of such extension in peptides used *in vitro*.

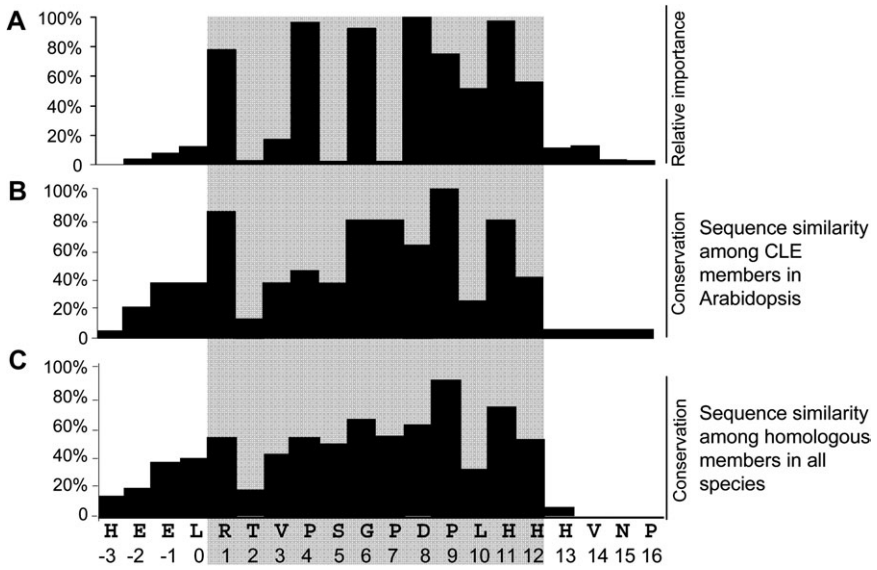

Figure 5. Comparison between Contributions of Individual Residues and Sequence Conservations.
(A) Summary of the relative contributions of individual residues in the core CLE motif and its flanking sequences to the CLV3 function in SAMs.
(B) Residue conservation among 31 CLE members in the *Arabidopsis* genome.
(C) Residue conservation among 213 putative peptide ligands found in all species, with sequence similarities to CLV3.
Note that the contributions of individual AA residues to the CLV3 function are not correlated to their conservations. Shaded, the core CLE motif.

Similarly, in the *in vitro* tracheary element differentiation assay performed in *Zinnia elegens* for synthetic TDIF, a homolog of CLE41 and CLE44, Ito et al. (2006) also observe that the terminal residue N12 is critically important. Interestingly, the 12th residue is almost exclusively occupied by H or N in 213 peptide ligand-related family members including CLE, AtPep1, and RGF found in *Arabidopsis*, poplar, rice, wheat, soybean, nematode, and *Physcomitrella patens* (supplementary data). It is plausible that the substitution of H12 by alanine may lead to reduced stability of the synthetic CLV3 peptide *in vitro* and then the activity of the peptide; while in the endogenous situation, the peptide may be produced continuously.

The importance of the residue D8 has essentially been detected in our *in vivo* complementation assay, not in previous *in vitro* assays. Among all CLE members known, the eighth position is exclusively occupied by either a negatively charged aspartatic acid (D) or an uncharged asparagine (N) (Oelkers et al., 2008). CLE members with N at the eighth residue, such as CLE9 and CLE13, are able to partially complement the *clv3-1* defects when expressed under the control of *CLV3* regulatory elements (Ni and Clark, 2006). A more recent paper showed that the replacement of D8 by N causes only minor damage to CLV3 in complementing *clv3-2* (Ni et al., 2011). However, in our study, replacement of D8 by the smaller-molecular-weight non-polar alanine showed an almost complete loss of the function of CLV3, suggesting the conformation, not the charges, of this particular residue is important. In contrast, the importance of the G6 residue is observed in all these assays. This is consistent with genetic analyses. Severe mutant phenotypes have been observed in *clv3-1* and *clv3-5* that have point mutations in the G6, converting it to D and A, respectively (Fletcher et al., 1999; Ni et al., 2011). As the smallest and the free-rotating residue (Figure 5C), the sixth G is also conserved in AtPep1 family members (supplementary data) and shown to be important in AtPep1 in triggering innate immune responses (Pearce et al., 2008). Most likely, the free-rotating residue G located in the central region of these peptides plays an important role in maintaining their conformational flexibilities.

Alignment among CLE and other homologous members in different species showed that most residues, except the second and 10th ones, within the core CLE motif and three residues adjacent to the left border of the motif are conserved (Figure 5C and supplementary data). Our *in vivo* complementation studies showed that contributions of these residues to the CLV3 function did not correlate with their evolutional conservations (Figure 5). In particular, the relative conserved third and fifth residues and the previously assigned glycosylation residue P7 were not important for CLV3. Most likely, these conserved but non-essential residues may function as the core backbone for these peptides, while the specificity of a particular peptide may be created by other residues. For example, the moderately conserved P4 and D8 are critical for the CLV3 function. Since the modifications on P7 are detected only in seedlings or cell lines overexpressing the *CLV3* gene (Kondo et al., 2006; Ohyama et al., 2009), it is worth elucidating whether they indeed occur *in vivo* and, if yes, what their functions are.

The importance of sequences flanking the core CLE motif remains controversial. Domain deletion experiment shows that, in addition to the signal peptide, the 14-AA CLE motif and four AAs at its left border are sufficient to perform the CLV3 function in *Arabidopsis* (Fiers et al., 2006). However, domain-swapping experiments performed among CLE members show that sequences flanking the CLE motif contribute to the CLV3 function (Meng et al., 2010). A recent report shows that four residues (L0, E-1, E-2, and H-3) located at the left

border of the core CLE motif of CLV3 are important for the peptide cleavage (Ni et al., 2011). Our present studies showed that substitutions of L0, E-1, E-2, and H-3 flanking the left border and H13, V14, N15, and P16 flanking the right border of the core CLE motif of CLV3 with alanine only affected slightly the complementation efficiencies in *clv3-2 in vivo* (Figure 2B). Thus, how the CLV3 cleavage is regulated remains to be elucidated.

In summary, our *in vivo* complementation analyses in SAMs in combination with previous *in vitro* peptide assays in RMs (Kondo et al., 2008) reveal that shoot and root meristems may have distinct specificity requirements to peptide ligands. Additionally, the differences between endogenous CLV3 and *in vitro*-applied synthetic CLV3 peptide may reflect the difference in peptide processing and stability, which need to be elucidated in the future. The establishment of such an accurate contribution map for each residue in the peptide-coding region of CLV3 may help to elucidate the interactions between CLV3 peptide and its receptors for stem cell maintenance, and even functions of peptide hormones in general.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotypes Colombia-0 (Col-0), Landsberg *erecta* (L*er*), and *clv3-2* (in L*er* background) were grown in a growth room with 16 h of light per day at 21 ± 1°C. Seeds were surface-sterilized for 2 h in a desiccator with chlorine gas produced by mixing 50 ml of kitchen bleach (containing 5% sodium hypochlorite) and 5 ml of concentrated HCl. Transformation was performed via an *Agrobacterium tumefaciens*-mediated floral dip method (Clough and Bent, 1998). Transgenic plants were obtained under the selection with 25 mg L^{-1} glufosinate ammonium (Sigma, Shanghai).

Construction of Alanine-Substituted Constructs

A 3934-bp genomic fragment of *CLV3* was amplified from L*er* genomic DNA with primer pairs of CLV3–G-FP and CLV3–G-RP (Supplemental Table 1). This fragment was cloned into the *pDONR221* Gateway vector with BP Clonase II enzyme (Invitrogen, Beijing) to produce *pDONR221–CLV3* and was subcloned into the expression vector *pBGWFS7* (Karimi et al., 2002), generating a destination construct *pBGWFS7–CLV3*. For alanine substitutions, nested PCR was performed to introduce point mutations with a site-directed mutagenesis kit (Transgen, Beijing). The PCR product was used to replace the corresponding fragment in *pDONR221–CLV3* by *Hin*dIII digestions, and then cloned into *pBGWFS7* by Gateway technology (Invitrogen, Beijing). All primers used are listed in Supplemental Table 1.

In vivo Complementation Assays

Constructs carrying alanine-substituted full-length *CLV3* genomic DNA described above were transformed to *clv3-2* mutant. Six siliques in the bottom, middle, and top of 25 or more T1 transgenic plants were selected for carpel number analyses. For SAM area measurements, T2 plants derived from two to three T1 transgenic lines were analyzed as previously described (Fiers et al., 2006).

Real-Time Quantitative RT–PCR Analyses

An RNA isolation kit (Tiangen, Beijing) was used to extract the total RNAs from shoot apices excised from 30–40 T^2 plants of *clv3-2* transformed with constructs carrying alanine-substituted *CLV3*. Real-time quantitative RT–PCR (qRT–PCR) was performed with a Rotor-Gene 3000 thermocycler (Corbett Research, Shanghai) with the SYBR Premix ExTaq II kit (Takara, Beijing). The relative expression levels were normalized against *EIF4A* through the use of a modified $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). The primers used are listed in Supplemental Table 1.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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