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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.