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Short Communication

Gain-of-Function Phenotypes of Chemically Synthetic CLAVATA3/ESR-Related (CLE) Peptides in *Arabidopsis thaliana* and *Oryza sativa*

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Using 26 chemically synthetic CLAVATA3/ESR (CLE) peptides, which correspond to the predicted products of the 31 *Arabidopsis CLE* genes, we investigated the CLE peptide function in *Arabidopsis* and rice. Treatment with some CLE peptides inhibited root elongation in rice as well as in *Arabidopsis*. It also reduced the size of the shoot apical meristem in *Arabidopsis* but not in rice. Database searches revealed 47 putative CLE genes in the rice genome and multiple CLE domains in some CLE genes, indicating diverse CLE function in these plants.

Keywords: *Arabidopsis thaliana* — CLE peptides — *Oryza sativa* — Shoot apical meristem.

Abbreviations: CLE, CLAVATA3/ESR-related; LRR, leucine-rich repeat; RAM, root apical meristem; SAM, shoot apical meristem; TDIF, tracheary element differentiation inhibitory factor.

Accession numbers OsCLE101, AB332048; OsCLE102, AB332049; OsCLE103, AB332050; OsCLE104, AB332051; OsCLE201, AB332052; OsCLE202, AB332053; OsCLE203, AB332054; OsCLE204, AB332055; OsCLE205, AB332056; OsCLE206, AB332057; OsCLE301, AB332058; OsCLE302, AB332059; OsCLE303, AB332060; OsCLE304, AB332061; OsCLE305, AB332062; OsCLE306, AB332063; OsCLE401, AB332064; OsCLE402, AB332065; OsCLE403, AB332066; OsCLE404, AB332067; OsCLE501, AB332068; OsCLE502, AB332069; OsCLE503, AB332070; OsCLE504, AB332071; OsCLE505, AB332072; OsCLE506, AB332073; OsCLE507, AB332074; OsCLE508, AB332075; OsCLE509, AB332076; OsCLE601, AB332077; OsCLE602, AB332078; OsCLE603, AB332079; OsCLE701, AB332080; OsCLE801, AB332081.

Intercellular communication is a fundamental mechanism for coordinating the development of complex bodies of multicellular organisms such as plants and animals. In plant morphogenesis, *CLAVATA* (*CLV*) genes are key players for meristem formation. *CLV1*, *CLV2* and *CLV3* encode a leucine-rich repeat receptor-like kinase (LRR-RLK), a LRR receptor-like protein without the kinase domain, and a putative peptide, respectively (Clark et al. 1997, Fletcher et al. 1999, Jeong et al. 1999). CLV3 belongs to the CLV3/ESR-related (CLE) gene family that shares significant homology in 14 amino acids at the C-terminal region and has 31 *CLE* members in the *Arabidopsis* genome (Cock and McCormick 2001, Olsen and Skriver 2003, Sharma et al. 2003, Kondo et al. 2006). CLV3 and other CLE peptides are suggested to function in plant morphogenesis as intercellular signaling molecules (Chu et al. 2006, Fiers et al. 2006, Ito et al. 2006, Ni and Clark 2006, Strabala et al. 2006, Suzaki et al. 2006).

We have identified that tracheary element differentiation inhibitory factor (TDIF) and CLV3 encode dodecapeptides with two hydroxyproline residues, regulating vascular development and meristem formation, respectively (Ito et al. 2006, Kondo et al. 2006). Chemically synthesized TDIF and CLV3 peptides also function in our in vitro bioassay systems (Ito et al. 2006, Kondo et al. 2006, Sawa et al. 2006, Fukuda et al. 2007). In order to investigate the CLE peptide function in different plant species, we utilized 26 *Arabidopsis* CLE peptides corresponding to 31 *Arabidopsis* CLE gene products, as we used before (Ito et al. 2006).

Arabidopsis seeds were germinated on vertical plates with media containing individual peptides at 1 μM, and root length was observed at 8, 11 and 14 d after germination. Treatments with CLE1/3/4, CLE2, CLE5/6 and CLE7 did not affect root elongation, but CLE41/44, CLE42 and CLE46 enhanced it to a slight degree (Fig. 1A). On the other hand, the other 19 peptides reduced the size of the root apical meristem (RAM) (Supplementary Fig. S1) and also reduced the root growth rate, resulting in a short root phenotype (Fig. 1A; Supplementary Table S1).

To investigate the function of CLE peptides on shoot apical meristem (SAM) formation, *Arabidopsis* seedlings were grown in liquid medium with each of 26 CLE peptides at 1 μM. In order to clarify the effects of the peptides on

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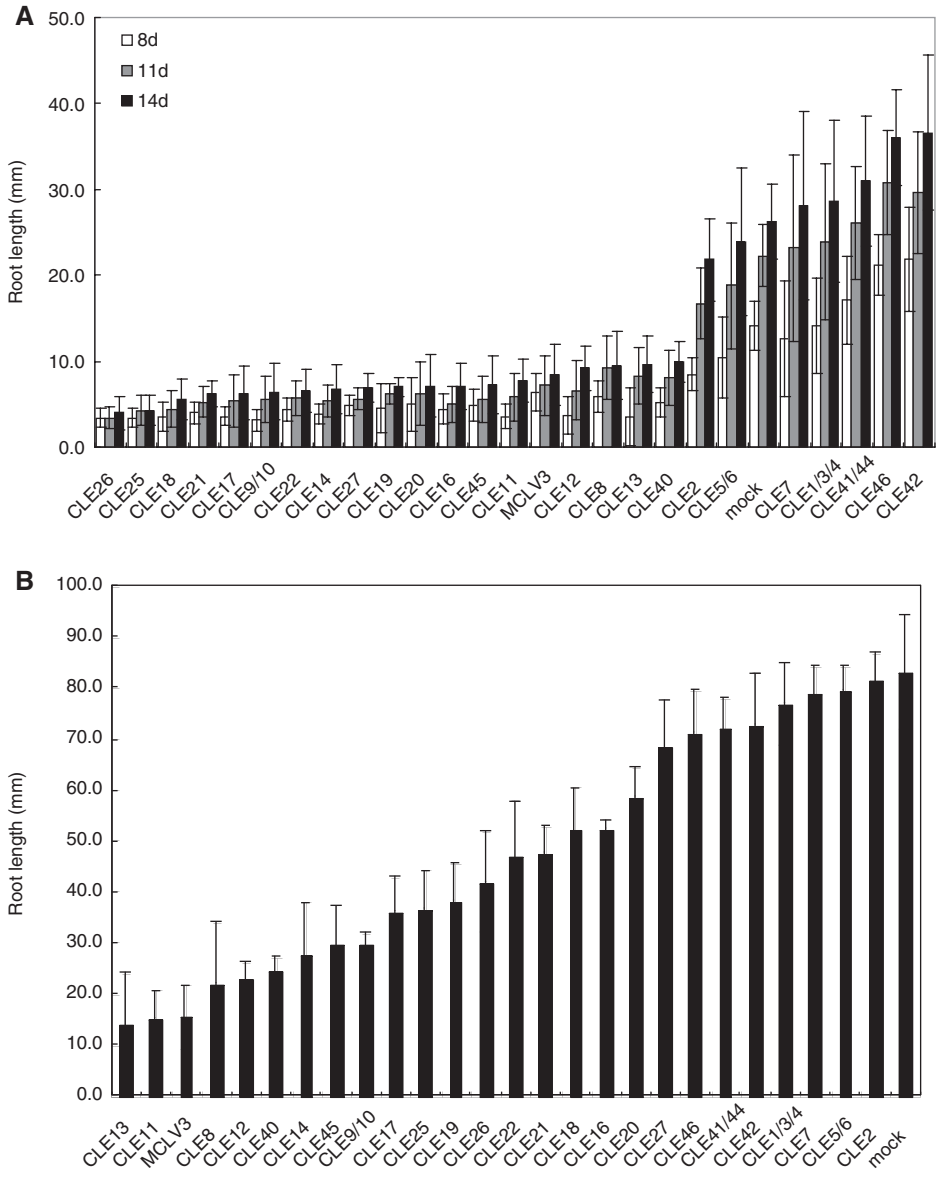

Fig. 1 CLE peptides affect *Arabidopsis* and rice root elongation. (A) *Arabidopsis* root length was measured at 8, 11 and 14 d after germination. Mean values and standard deviations were calculated by using at least seven samples. (B) Histograms of mean values of rice root length and standard deviations were calculated from at least eight samples grown on agar medium containing 1 μM of various CLE peptides grown for 2 weeks.

SAM size regulation, the SAM region was observed by using scanning electron microscopy, and we could classify the SAM structures into four types, floral SAM, vegetative-like SAM, flat SAM and V-shaped SAM (Fig. 2). When grown in control medium for 20 d after germination, 13 of 14 plants produced floral meristems (Fig. 2A, I). Treatment with the CLV3 peptide reduced the size of the SAM, showing a flat SAM with small leaf primordia (Fig. 2C, K) or a V-shaped SAM (Fig. 2D, L). CLE9/10, CLE11, CLE16, CLE18, CLE19, CLE25, CLE26, CLE40 and CLE45 also reduced the SAM size (Fig. 2G, H) similarly to the effects of plants receiving CLV3 peptide treatment, indicating that these peptides have strong effects on SAM size regulation. On the other hand, CLE1/3/4, CLE5/6, CLE7, CLE41/44, CLE42 and CLE46 did not prevent SAM formation (Fig. 2E), as was the case with the control treatment. Plants treated with CLE2, CLE8, CLE12, CLE13, CLE14, CLE17, CLE20, CLE21, CLE22 and CLE27 produced vegetative-like SAMs (Fig. 2B, F, J; Supplementary Table S1), indicating that these peptides

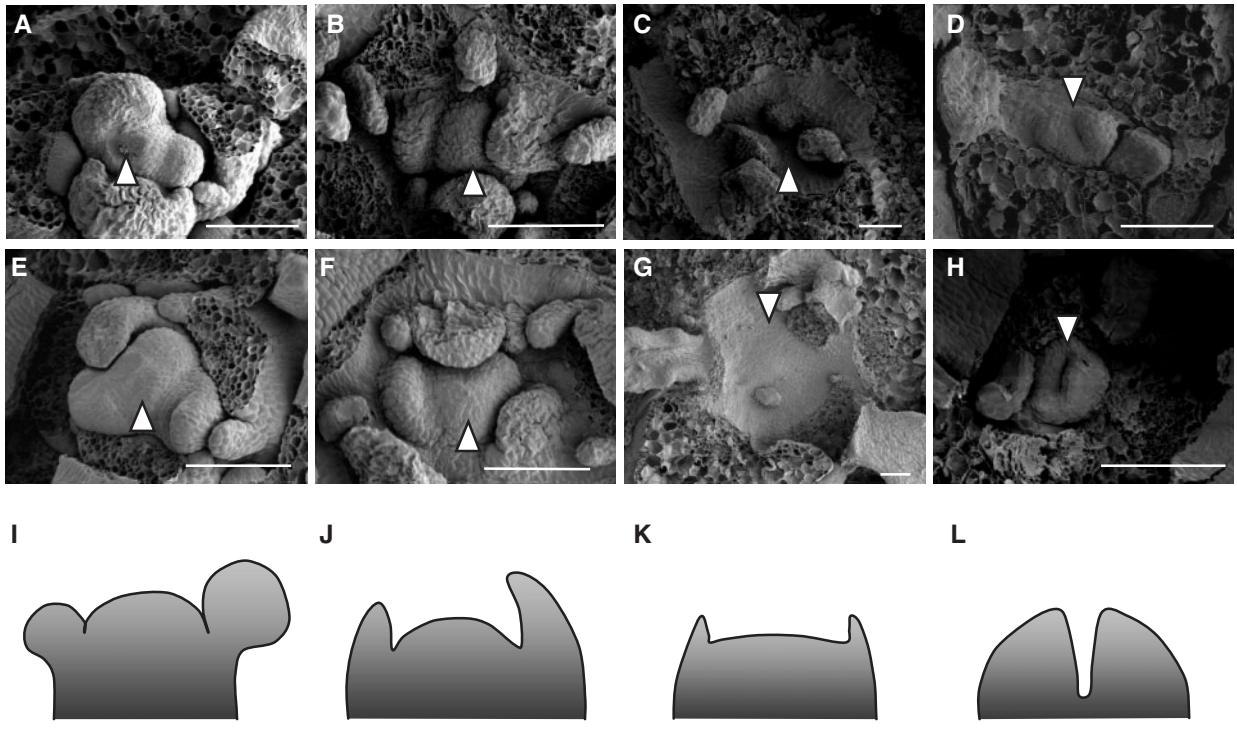

Fig. 2 CLE peptides affect *Arabidopsis* SAMs. The *Arabidopsis* SAM region is indicated by white arrowheads. Wild-type plants were grown in liquid medium and sampled at 20 d after germination. The plants were treated with (A) control, (B) CLE12, (C, D) CLV3, (E) CLE1, (F) CLE8, (G) CLE11 and (H) CLE25. (I–L). Schematic drawing of (I) floral SAM, (J) vegetative-like SAM, (K) flat SAM and (L) V-shaped SAM. Bars: 100 μm.

have mild SAM-repressing effects or a function in inhibition of flowering induction.

Oryza sativa is well known as a model organism of monocotyledonous plants, and sequencing of the entire rice genome has already been completed. Genes encoding putative CLE domains were sought in the RAP-DB (http://rapdb.lab.nig.ac.jp/), with *Arabidopsis* CLE sequences of 12 amino acid residues as queries. In total, 47 putative *OsCLE* genes were found in the rice genome. The rice genome has more *CLE* genes than *Arabidopsis*, and they are scattered throughout the genome. As has been noted previously (Sharma et al. 2003), there is little sequence conservation among the *CLE* genes, except the CLE domain. A phylogenetic tree was produced with 12 amino acid sequences corresponding to functional CLE peptide sequences (Ito et al. 2006, Kondo et al. 2006), and it revealed that most of the rice *CLE* genes are classified in a similar fashion to *Arabidopsis CLE* genes (Supplementary Fig. S2). However, two new clades were found containing the peptide sequences OsCLE501, OsCLE502A and OsCLE502B, and OsCLE506A–OsCLE506E, respectively (Supplementary Fig. S2). These genes may be responsible for rice-specific events. In rice, three genes, OsCLE502, OsCLE504 and OsCLE506, encode multiple CLE domains, although no gene encodes multiple CLE domains in *Arabidopsis*.

In order to investigate the CLE function in rice, we treated wild-type seeds of *O. sativa*, Taichung 65, with 1 μM of the 26 *Arabidopsis* CLE peptides in agar medium. Crown root length was measured 14 d after germination. CLE41/44, CLE42 and CLE46 weakly repressed root elongation (Fig. 1B). Treatment with CLE1/3/4, CLE2, CLE5/6 and CLE7 did not affect root growth, and the other 19 peptides inhibited root elongation (Figs. 1B, 3A–D). Although *Arabidopsis* CLE peptides affected the rice RAM in a similar fashion to *Arabidopsis*, CLE-treated rice plants showed more gradational inhibitory effects on root elongation, in contrast to their severe inhibition in *Arabidopsis* (Fig. 1A, B; Supplementary Table S1). These results may suggest that some classes of peptides showing a weaker effect on RAM activity are involved in events other than root growth in rice, or that some *Arabidopsis* peptides can bind less effectively to the rice receptors due to the sequence differences between *Arabidopsis* and rice counterparts.

On the other hand, none of the 26 CLE peptides at 1 and 5 μM affected the morphology of the rice SAM in our liquid culture system (Fig. 3E, F). Chu et al. (2006) showed that the FLORAL ORGAN NUMBER2 (FON2)/FON4 peptide induced a dwarf seedling phenotype and a reduced SAM size in rice. In order to examine other rice CLE peptide functions, we investigated the effects of chemically

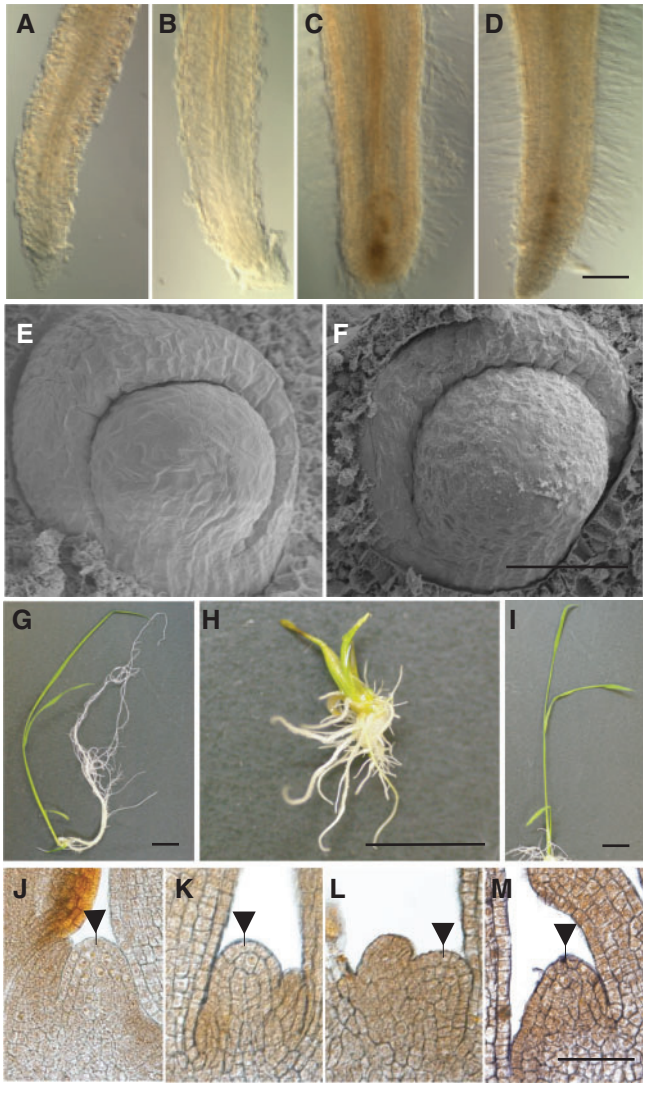

Fig. 3 Effects of CLE peptides on rice RAMs and SAMs. Wild-type Taichung 65 grown in agar medium (A–D) and in liquid medium (E–M) treated with control (A, E, G, J), 1 μM CLE1/3/4 (B), CLV3 (C, 1 μM; F, 5 μM; M, 100 μM), 1 μM CLE13 (D), OsCLE202 (H, 100 μM; K, 50 μM; L, 100 μM) and 100 μM CLE5/6 (I) peptides. Root hairs were produced even at the root tip (C, D). Arrowheads indicate the SAM region (J–M). Bars: 1 cm (G–I), 100 μm (A–F, J–M).

synthesized rice peptides of OsCLE202. This was because OsCLE202 is the only rice peptide that belongs to the clade of CLE8, CLE11, CLE12 and CLE13 (Supplementary Fig. S2), and these peptides are most effective for rice RAM size regulation. In addition, CLE11 showed strong SAM size reduction in *Arabidopsis*. OsCLE202 peptide treatment induced short root and severe dwarf phenotypes (Fig. 3G, H), but the SAM structure was not so severely affected in rice (Fig. 3J–L). As a control, we used CLV3 peptide and CLE5/6 peptide, that did not affect *Arabidopsis* and rice RAM size. CLV3 peptide induced the dwarf phenotype as in the case of OsCLE202 peptide treatment, and the CLV3 peptide did not affect the rice SAM size even at high concentration (Fig. 3M). In contrast, CLE5/6 peptide did not induce the dwarf phenotype (Fig. 3I). These results suggested that some rice CLE peptides, including OsCLE202, may be responsible for the growth of aboveground tissue, and that SAM size in rice is regulated by a different class of CLE peptides from those in *Arabidopsis*.

In our study, 19 CLE dodecapeptides were functional in *Arabidopsis* RAM size regulation, suggesting that CLE dodecapeptides act on RAM size regulation in vivo in a similar manner to the action of the CLV3 peptide on SAM size regulation. Regarding SAM regulation, only 10 CLE peptides exhibited a strong effect. This indicates that CLE peptides function less redundantly in the SAM than in the RAM. Interestingly, a typical acidic amino acid, D8, is conserved in six out of 10 CLE peptides that showed strong effects on SAM regulation. Considering that the substitution of alanine for N8 caused a severe loss of TDIF activity (Ito et al. 2006), the polarity and acidity of the eighth amino acid of the CLE peptides might have a critical function as a ligand in SAM size regulation.

Nineteen CLE peptides clearly function in *Arabidopsis*, whereas they showed gradational effects on short root phenotypes in rice. Furthermore, OsCLE202 induced not only short root phenotypes, but also the dwarf phenotype, which indicates the dual function of the OsCLE202 peptides in size regulation in the RAM and aboveground tissues. On the other hand, the CLE18, CLE25 and CLE26 peptides are most effective in RAM size regulation in *Arabidopsis*, whereas plants in which these genes were overexpressed showed long root phenotypes (Strabala et al. 2006). These results indicated that the CLE peptide function and structure, and the peptide–receptor combinations may have complex evolutionary diversity in various plants.

Forty-seven putative CLE genes are expected in the rice genome, and we found that three genes, OsCLE506, OsCLE502 and OsCLE504, encode multiple CLE domains. Yeast α factor is a peptide pheromone of 13 amino acids involved in the mating response of haploid cells, and four α factor copies are produced from one precursor (Dmochowska et al. 1987). It is suggested that multiple peptides, produced from one precursor, contribute to an efficient response in the mating process. Similarly, these rice CLE genes may function in some events in which rice needs a rapid response. The events may also be rice specific, because *Arabidopsis* does not have CLE genes that encode multiple CLE domains.

In conclusion, CLE genes have functional redundancy. Furthermore, we speculate that the CLE genes have functional diversity in each plant, and some plants might have unique CLE genes, probably for species-specific functions. Further genetic, biochemical and physiological

analyses would open up a new avenue to understanding not only the molecular mechanisms, but also the diversity and evolution of intercellular communication by CLE peptides in plants.

Materials and Methods

Columbia ecotype (*A. thaliana*) and Taichung 65 (*O. sativa*) were used as wild-type strains. Twenty-six CLE dodecapeptides were synthesized by OPERON with a purity of >95% and added to each medium (Kondo et al. 2006). For the peptide treatment on rice SAMs, Taichung 65 seeds were cultured in liquid growth medium containing a 1/2 concentration of Murashige and Skoog basal salts and 6% sucrose for 12 d with or without CLE peptides.

Scanning electron microscopy analysis was basically performed as described before (Sawa et al. 1999). Plant materials cultured in liquid medium were fixed with Carnoy's solution (acetic acid: ethanol = 1: 3) and then dehydrated and cleared through an ethanol series. The samples were frozen and dried (FDU-2100, EYELA, Tokyo, Japan), Pt-coated (JFC-1300, JEOL, Tokyo, Japan), and observed with a scanning electron microscope at 50 kV (JSM-820S, JEOL, Tokyo, Japan).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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