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Dual assay for MCLV3 activity reveals structure–activity relationship of CLE peptides

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ABSTRACT

The dodecapeptide MCLV3 is a functional peptide, derived from the CLV3 precursor protein, which is a candidate ligand of the CLV1/CLV2 receptor complex that restricts the stem cell population in the shoot apical meristem (SAM). MCLV3 can induce shoot and root meristem consumption, the typical phenotype of transgenic plants overexpressing *CLV3*. We investigated the bioactivities of a series of alanine-substituted MCLV3 and related peptides on the root growth of *Arabidopsis*. The structure–activity relationship (SAR) of MCLV3 had high similarity with that of tracheary element differentiation inhibitory factor (TDIF). We also evaluated the binding activities of the peptides by a competitive receptor binding assay using tritiated MCLV3 and the membrane fraction of a tobacco BY-2 cell line overexpressing the MCLV3 ectodomain. This dual assay, combining a biological and receptor binding assay for evaluating the activities of MCLV3-related peptides, uncovered the SAR of MCLV3, and indicated that the terminal residues play critical roles in exerting its activity and are important for specific binding to the receptor, CLV1.

All of the highly complicated and specialized organs of higher plants above ground, the leaves, stems, and flowers, are ultimately generated from a handful of stem cells in the shoot apical meristem (SAM). The number and the extent of differentiation of the stem cells are strictly controlled spatially and temporally through intricate cell-to-cell signaling.

The *CLAVATA* genes (*CLV1*, *2*, and *3*) play a crucial role in this process, and loss-of-function mutants of these genes accumulate stem cells on the SAM, resulting in a greatly enlarged dome-shaped meristem [1,2]. The *CLV1* gene encodes a leucine-rich repeat receptor-like kinase (LRR-RLK) and *CLV2* encodes a receptor-like protein (LRR-RLP) similar to CLV1 but without the kinase domain [3,4]. *CLV3* encodes a 96-amino acid small protein; the 18-amino acid NH_2-terminal hydrophobic region acts as a signal peptide to direct the protein into the secretory pathway [5,6]. The *CLV3* gene is a member of a large family of *CLE* (for *CLAVATA3/ESR-related*) genes that share a 14-amino acid highly conserved region located near the C-terminus (CLE motif) [7]. Synthetic 14-amino acid peptides that follow the CLE motif of CLV3, CLE19, and CLE40 can mimic the overexpression phenotype in *Arabidopsis* root meristem [8]. These data suggest that this conserved region is critically important for the *in vivo* function of CLE proteins.

We reported the identification of a peptide named MCLV3, which was detected from CLV3-overexpressed calli [9]. MCLV3 was derived from the 12 central residues of the CLE motif of CLV3, and its 4th and 7th proline residues were hydroxylated. The *in vivo* bioactivity evaluation of MCLV3 and related peptides indicated that MCLV3 is the shortest functional peptide derived from CLV3 precursor protein. The dodecapeptide named TDIF (tracheary element differentiation inhibitory factor) was coincidentally discovered from the culture broth of *Zinnia* mesophyll cells as an inhibitor of cell differentiation of tracheary elements [10]. The amino acid sequence of TDIF was identical with the deduced sequence of the CLE motif of the *TDIF* gene from *Zinnia* and *CLE41/44* from *Arabidopsis*, and two of the three proline residues were hydroxylated, in the same manner as MCLV3. These data suggest that the expressed CLE family precursor proteins are processed to the functional form and act as hydroxylated dodecapeptides *in vivo*. These modified dodecapeptides have been proposed to be called CLE peptides [10,11].

Recently, it was reported that the ectodomain of CLV1 is sufficient to constitute a specific binding site for MCLV3 [12]. This was the first evidence that MCLV3 directly binds to the CLV1 receptor. This result enabled us to evaluate the binding potential of MCLV3-related peptides with a biochemical method. In this report, we synthesized a series of MCLV3-related peptides, and evaluated the activity of each by a conventional root growth inhibition assay and a competitive receptor binding assay. The data obtained from the dual assay clarified the SAR of MCLV3 and the amino acid residues important for specific MCLV3 activity.

Materials and methods

General procedures. We performed electrospray ionization ion-trap mass spectrometry (ESI-IT-MS) using an Esquire 3000 instrument

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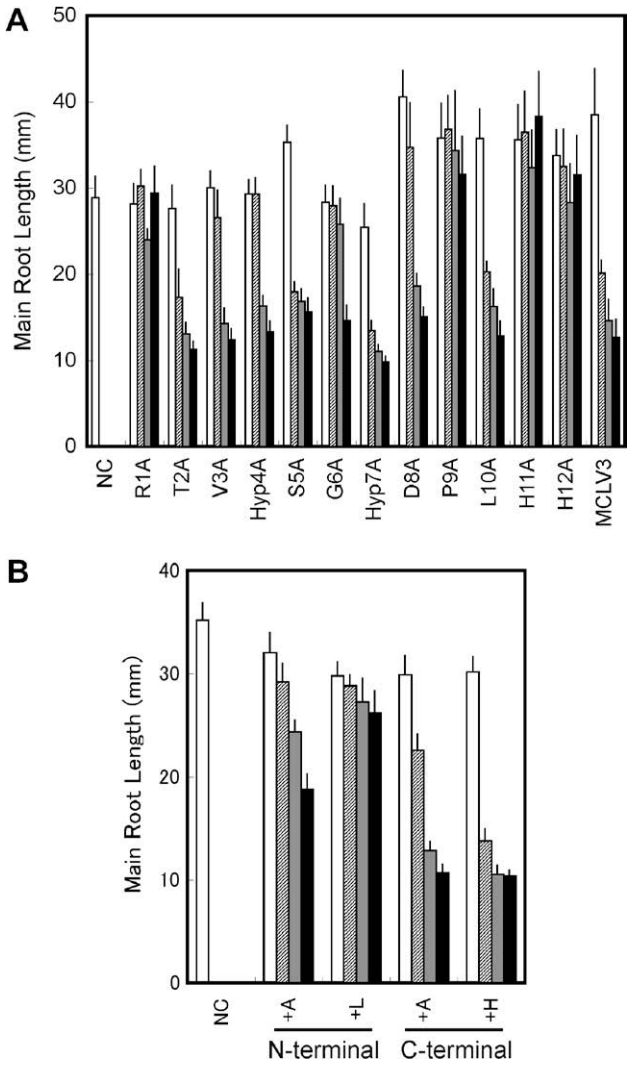

Fig. 1. Root growth inhibition assay of MCLV3-related peptides. (A) Alanine scan of MCLV3. (B) The bioactivity of N- and C-terminally elongated peptides. The peptides were added to the medium at a concentration of 1 nM (open bars), 10 nM (shaded bars), 100 nM (gray bars), or 1 μM (solid black bars). NC indicates non-peptide control. Each bar represents the average of 20 plants. Error bars indicate the standard deviation.

(Bruker Daltnics) equipped with an Agilent 1100 series HPLC system (Agilent Technologies). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MS/MS were performed with a 4700 proteomics analyzer (Applied Biosystems) using α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix.

Peptide synthesis and purification. All peptides were synthesized by Fmoc solid phase peptide synthesis using an ABI 433A peptide synthesizer (Applied Biosystems). Synthesized peptides were deprotected and cleaved from the resin with TFA cleavage cocktail containing 2-mercaptoethanol. The peptides were purified by reverse-phase HPLC with two solvent delivery pumps and a UV detector set at 220 nm. A Develosil-ODS HG-5 10 x 250 mm column coupled to a guard column (Nomura Chemical Co.) was used. Separation was carried out with a linear gradient of solvent A (H_2O/CH_3CN (98:2) with 0.5% of acetic acid) to solvent B (H_2O/CH_3CN (30:70) with 0.5% of acetic acid) as follows: 0% of B to 50% of B over 20 min, 50% B to 100% B over 5 min at 3.0 ml/min. The purity of each peptide was assessed by its UV spectrum (210 nm) and extracted ion chromatogram of LC–MS analysis.

Root growth inhibition assay. Wild-type plants were *Arabidopsis thaliana* Columbia (Col) accessions. Plants were grown under constant fluorescent light at 23 °C. For the root growth inhibition assay, Col seeds were sown in a line on Gamborg's B5 agar medium [13] containing 0.7% agar plated in square petri dishes. Sown seeds were placed at 4 °C for 2–3 days and then the lower parts (under the sown seeds) of the dishes were masked with lightproof paper before incubation. The dishes were placed perpendicularly under the light and incubated for 10–12 days, then the lengths of the main roots were measured.

Receptor binding assay. We synthesized tritiated MCLV3 (73.3 Ci/mmol), and performed the receptor binding assay as described by Ogawa et al. [12]. The synthesized peptides were dissolved at −30 °C at a concentration of 10 mM in distilled water containing 0.1% trifluoroacetic acid (TFA). These peptide solutions were diluted with distilled water containing 0.1% TFA before use.

Results

Alanine scanning through root growth inhibition assay

We synthesized MCLV3-based 12 peptides, each residue of which was alanine-substituted by Fmoc solid phase peptide synthesis. These alanine-substituted peptides are abbreviated R1A, T2A, V3A, Hyp4A, S5A, G6A, Hyp7A, D8A, P9A, L10A, H11A, and H12A. After deprotection and reversed-phase HPLC, we estimated the purity of the peptides by LC–MS analysis.

Since MCLV3 and a group of CLE peptides consume the root meristem and thus inhibit growth of the main root of *Arabidopsis* at nanomolar concentrations [8,9], the bioactivities of the alanine-substituted peptides were estimated by an *Arabidopsis* root growth inhibition assay that features high sensitivity and quantitative capability for MCLV3-related peptides. Fig. 1A shows the root growth inhibition activities of the alanine-substituted peptides. MCLV3 inhibited root growth at 10 nM, and the activity was unchanged above 100 nM. Alanine substitution at positions 1, 9, 11, and 12 resulted in complete loss of activity, demonstrating the significance of these residues for expressing the inhibitory activity on the roots. Since V3A, Hyp4A, and D8A showed clear activity at 100 nM, which is a concentration 10 times higher than that of MCLV3, these residues appeared to be important, but not critical, for MCLV3 function. Despite the substitution by a structurally related amino acid residue, G6A was 100 times weaker than MCLV3. The four peptides, T2A, S5A, Hyp7A, and L10A, showed the same profile as that of MCLV3, which suggested that these residues play a small, if any, role in exerting MCLV3 activity on the roots.

We also synthesized four peptides to which an alanine or the original amino acid in the CLV3 precursor protein was added at the N- or C-terminus of MCLV3, and evaluated root growth inhibition activity of these (Fig. 1B). Amino acid addition at the N-terminus caused a loss of bioactivity, whereas the bioactivity of C-terminally elongated peptides was as strong as that of MCLV3.

Swapping experiment between MCLV3 and TDIF

An alanine scan study of TDIF in a previous report [10] indicated that positions 1, 3, 4, 6, 8, 9, 12 are important for TDIF inhibitory activity on tracheary element differentiation (Fig. 2A). This result shows very good agreement with our alanine scan result except for that of the 11th residue. As mentioned above, *CLV3* and *TDIF* belong to a large *CLE* gene family that is defined by high homology of the *CLE* motif at the C-terminus. This observation indicates that MCLV3 possesses a high sequence similarity to TDIF; however, MCLV3 activity and TDIF activity do not show any crossover: all tested CLE peptides that show MCLV3-like root growth inhibition activity do not show any TDIF activity, and *vice versa* [10]. This result suggests that the specific residues that endow the individual character of each peptide are in these two peptides. An amino

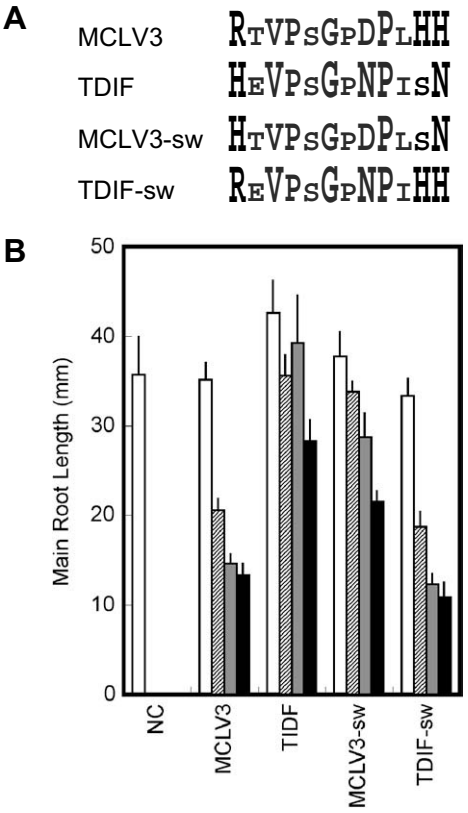

Fig. 2. Swapped peptides show importance of individual residues in root inhibition. (A) The amino acid sequences of MCLV3, TDIF and the swapped peptides. The essential residues for root growth inhibition (MCLV3) or inhibition of cell differentiation of tracheary elements (TDIF) are indicated by the largest letters. The important (but not essential) residues are indicated with the medium-size letters. (B) Root growth inhibition assay of swapped peptides. The peptides were added to the medium at a concentration of 1 nM (open bars), 10 nM (shaded bars), 100 nM (gray bars), or 1 μM (solid black bars). NC indicates non-peptide control. Each bar represents the average main root length of 20 plants. Error bars indicate the standard deviation.

acid sequence comparison of MCLV3 and TDIF shows that these peptides are highly homologous from the 2nd to the 10th residue, and especially, that the residues that are important for exerting bioactivity most closely resemble each other. On the other hand, the amino acids at positions 1, 11, 12 are quite different in spite of their significance on exerting the respective activity. Therefore, a swapping experiment between MCLV3 and TDIF was undertaken to determinate the significance of these terminal residues in specifying MCLV3 activity.

The bioactivities of synthesized swap peptides were evaluated using a root growth inhibition assay (Fig. 2B). MCLV3-sw, which is composed of the MCLV3-type central region and TDIF-type termini showed quite weak inhibitory activity, whereas TDIF-sw, which has a TDIF-type central region and MCLV3-type termini showed clear inhibitory activity, as strong as MCLV3. These data suggest that the characteristic terminal region plays a key role in specifying the function of MCLV3 and TDIF, whereas the contribution of the exchangeable central region to specificity is relatively small.

Receptor binding assay

To assess the binding potential of the synthetic peptides, we performed a competitive assay using [^{3}H]MCLV3 and a membrane fraction of the tobacco BY-2 cell line containing a recombinant protein of the CLV1 ectodomain fused with HaloTag protein (CLV1-ΔKD-HT) [12].

The competitive binding assays were performed using 2 nM [^{3}H]MCLV3 and a 100-times higher concentration of the alanine-substituted peptides (Fig. 3A). Under these experimental conditions, R1A and H12A did not show any competition for binding. V3A, Hyp4A, G6A, D8A, and H11A partially competed for binding sites, indicating that these peptides weakly bind to CLV1-ΔKD-HT. On the other hand, T2A, S5A, Hyp7A, and L10A showed strong activity in the root growth inhibition assay, inhibiting the specific binding of [^{3}H]MCLV3 completely. Taken together, the profile was similar to that of the root growth inhibition assay (Fig. 1A) except for P9A, which was a strong competitor despite complete loss of bioactivity against the roots. When an excess amount of the peptides (2 μM) was added in the competition assay, the R1A and H12A peptides did not compete for MCLV3 specific binding sites, and the other peptides almost completely inhibited specific binding competitively (data not shown).

We also tested the binding potential of terminally elongated or swapped peptides under the same conditions. The results of the competition assay suggested that all elongated peptides weakly bind to CLV1-ΔKD-HT, and the binding potential of C-terminally elongated peptides was stronger than that of N-terminally elongated peptides (Fig. 3B). Among the swapped peptides, TDIF-sw strongly bound to the receptor, whereas MCLV3-sw and TDIF showed little or no competition (Fig. 3C), which suggests that the differences in root growth inhibitory activity of the swapped peptides arise from their difference in affinity to CLV1.

Discussion

After the discovery of the *CLV3* and *CLE* gene family, an increasing number of *CLE* genes have been identified in the last decade using bioinformatics-based approaches [7,14–16]. An intensive search for unidentified *CLE* genes revealed that *CLE* genes are encoded in the genome of many plants, from higher plants to a moss and a green alga [17]. CLE peptides are suggested to be plant-specific; the only exception has been found in nematodes [18–20]. Up to now, 179 genes have been identified as *CLE* genes and the number is expected to increase in concurrence with the enhancement of plant genomic resources.

In some recent reports, genetic approaches were used for understanding and predicting the *in vivo* functions of these *CLE* genes. Overexpression or controlled expression of *CLE* genes leads to curious phenotypes; one phenotype similar to *CLV3* overexpression, another dwarf phenotype without effect in SAM function, another overriding *clv3* phenotype [15,21]. These data give a meaningful suggestion for presuming the *in vivo* function of *CLE* genes; however, it is difficult to figure out the exact contribution of the resulting CLE peptides for each biological step without quantitation of the bioactive peptides *in vivo*, whereas bioassay using synthetic peptides as described in this report enables the quantitative evaluation of the bioactivity of the peptides. This approach is thought to aid in evaluating the function of CLE peptides and CLE genes from the other point of view, and a combination of these approaches will lead to understanding the real function of the *CLE* genes and CLE peptides.

The alanine scanning experiment on MCLV3 revealed that the 12 residues of MCLV3 can be classified into three categories depending on their contributions to MCLV3 activity on *Arabidopsis* root growth. The first category consists of ^{1}Arg, ^{9}Pro, ^{11}His, and ^{12}His. These irreplaceable amino acids are located near each end of the peptide, which agrees with previous results that N- or C-terminally truncated MCLV3 did not show any activity against root and shoot meristems [8,9]. The second category includes ^{3}Val, ^{4}Hyp, ^{6}Gly, and ^{8}Asp. These residues are expected to play important roles in MCLV3 function, but their contributions are not essential. It was reported that the glycine residue would play a pivotal role in CLV3

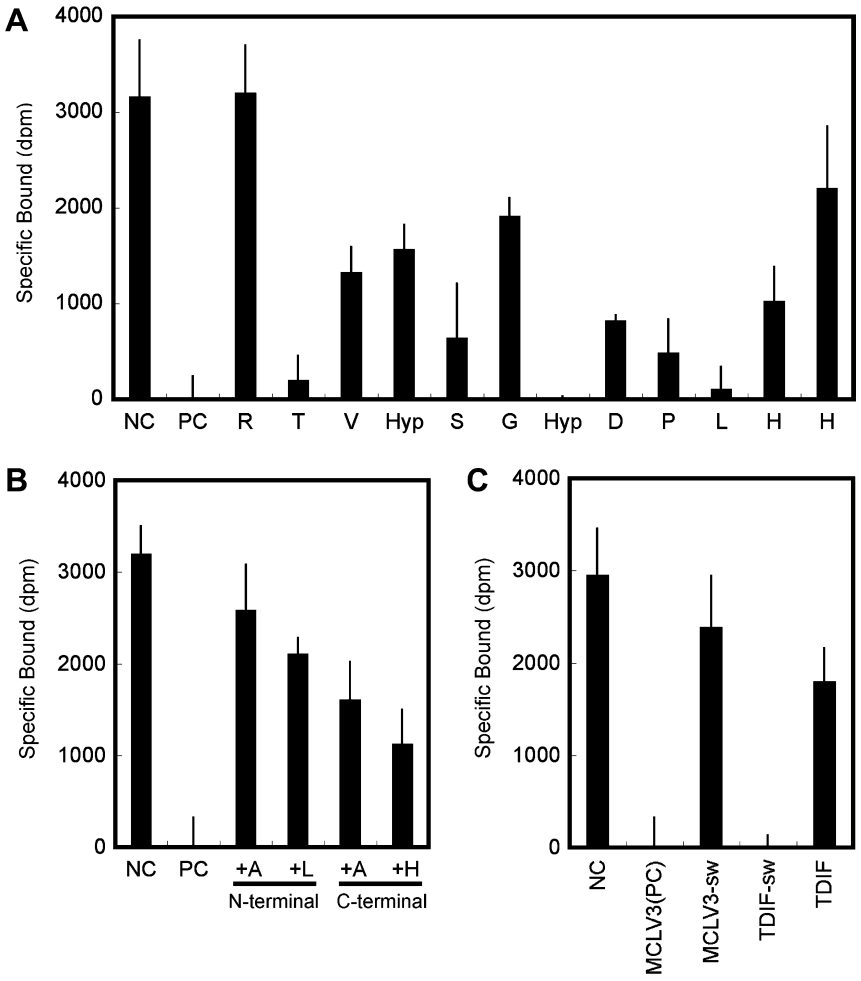

Fig. 3. Competitive receptor binding assay. (A) Alanine scan of MCLV3. (B, C) N- and C-terminally elongated peptides and swapped peptides. Each bar represents the tritium count of [^{3}H]MCLV3 specifically bound to CLV1-ΔKD-HT. NC and PC respectively indicate non-peptide and positive (MCLV3-added) control. All data represent the average of triplicate experiments. Error bars indicate the standard deviation.

function because a point mutation at this residue was found in two independently derived loss-of-function mutant lines of *CLV3* (*clv3-1* and *clv3-5*) [5]. The G6A peptide showed about 100-times weaker activity on the roots in spite of a slight change in structure, which supports the previous data based on mutant analysis. The last category consists of ^{2}Thr, ^{5}Ser, ^{7}Hyp, and ^{10}Leu, of which the corresponding alanine derivatives exerted as strong activity as that of MCLV3. These residues probably can be substituted by any proper amino acid possessing a suitable functional group, which can then be used as a scaffold to construct a variety of molecular probes for photoaffinity labeling or fluorescent tracer studies without affecting its activity.

The results with N- and C-terminally elongated peptides indicate that N-terminal elongation decreases the activity in spite of the addition of the amino acid originally encoded in *CLV3* gene. In the case of acetylation of the N-terminal amino group, activity was practically lost in the root growth inhibition assay (data not shown). Strict specificity of N-terminal recognition implies the importance of exposure of the N-terminal arginine and the terminal amino group for receptor binding. In contrast, C-terminal elongation did not affect root growth inhibitory activity, whereas the receptor binding potential of C-terminally elongated peptides was relatively weak. The low specificity at the C-terminus suggests that maturation at the C-terminus is due to the existence of a nonspecific, endo-type protease in the apoplastic fluid, which was implied in a previous report [22]. This model can explain the result that the C-terminally elongated peptides showed as strong activity as MCLV3 even though their binding potential was lower than that of MCLV3, because in the root growth inhibition assay, it takes a long time for the added peptides to be digested by the enzyme that converts the elongated peptides into active MCLV3 *in vivo*.

The swapping experiments revealed that the central region (^{2}Thr to ^{10}Leu) is exchangeable between MCLV3 and TDIF. The residues that are important for each activity in this region are highly conserved in the amino acid sequences of 26 independent CLE domains of *Arabidopsis* [7,10]. In addition, alanine-substitution experiments indicate that the contribution of the residues in this region is important but not essential. We speculate that this central region acts as a common backbone to fix the peptide in the bioactive conformation.

Since the expression of CLV1 is restricted to the organizing center in the SAM, a root-specific unidentified receptor that acts as a heterodimer with CLV2 receptor-like protein is assumed to be activated when one of a series of CLE peptides is added to the culture broth [8]. Despite some exceptions, the results of the three assays, the root growth inhibition assay, the competitive receptor binding assay, and the TDIF assay of a previous report [10], showed good agreement with each other, which indicates that CLV1 and the unidentified receptors, namely the TDIF receptor and the root-specific CLE peptide receptor, share similar characteristics of binding to each ligand.

It is noteworthy that the P9A peptide showed no effect on root growth but acted as a strong competitor in the receptor binding assay. The TDIF-related peptide, of which the highly conserved ^{9}Pro was replaced by alanine, did not show any TDIF activity, which implies that this proline residue is the common essential residue for bioactivity of CLE peptides. Accordingly, it is expected that P9A would bind to the receptor without activating it, which suggests that P9A could be used as a lead compound for an antagonist of MCLV3 activity. Thus, the basic information about SAR of MCLV3 described in this report will support the challenge of developing a practical agonist or antagonist of CLE peptides.

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