Analogs of the CLV3 Peptide: Synthesis and Structure–Activity Relationships Focused on Proline Residues

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(Received June 25, 2010; Accepted September 23, 2010)

Introduction

The CLAVATA3 (CLV3) peptide is a secreted signaling molecule produced in the shoot apical meristem (SAM) of Arabidopsis thaliana. A loss-of-function clv3 mutant aberrantly accumulates stem cells in the SAM, resulting in the formation of increased numbers of flower organs and club-shaped siliques (Fletcher et al. 1999). The CLV3 precursor protein is post-translationally processed to be a glycosylated 13 amino acid peptide (Ohyama et al. 2009), which is derived from a 14 amino acid conserved motif called the CLV3/ESR-related (CLE) domain, located near the C-terminus of the precursor (Cock and McCormick 2001). In the course of searching for the mature, functional peptide of CLV3, we identified a dodecapeptide (MCLV3) from CLV3-overexpressing Arabidopsis calli; MCLV3 was the smallest functional peptide derived from the CLV3 precursor. Here, we designed a series of MCLV3 analogs in which proline residues were substituted with proline derivatives or N-substituted glycines (peptoids). Peptoid substitution at Pro9 decreased bioactivity without affecting specific binding to the CLV1-related protein in cauliflower membrane. These findings suggest that peptoid-substituted peptides would be lead compounds for developing potential agonists and antagonists of CLV3.

Keywords: Arabidopsis thaliana • CLV3 • Peptide hormone • Structure–activity relationship.

Abbreviations: ANOVA, analysis of variance; Boc, tert-butoxycarbonyl; CLE, CLV3/ESR-related; CLV3, CLAVATA3; Fmoc, 9-fluorenylmethyloxycarbonyl; MS, mass spectrometry; NMR, nuclear magnetic resonance; SAM, shoot apical meristem; SAR, structure–activity relationship; TDIF, tracheary element differentiation inhibitory factor; TFA, trifluoroacetic acid.
root growth inhibition assay and a competitive receptor binding assay (Ogawa et al. 2008), which uses tritiated MCLV3 and a membrane fraction prepared from the curd of cauliflower.

**Results**

**Evaluation of MCLV3 binding activity in cauliflower membrane fraction**

Several methods have been used to evaluate and quantify the potency of CLE peptides. Among these, the conventional root growth inhibition assay using Arabidopsis wild-type plants has been used for evaluating the biological activity of MCLV3 analogs because of its high sensitivity (Kondo et al. 2008). In addition to this method, we investigated the receptor binding potential of the analogs by using a competitive receptor binding assay (Ogawa et al. 2008). The combination of these two assays enabled us to discover not only agonists but also leading compounds for antagonists that can compete with endogenous CLV3 or other CLE peptides without having their own bioactivity.

In previous reports, the membrane fraction of a tobacco BY-2 cell line overexpressing the tagged CLV1 ectodomain (CLV1-ΔKD-HT) was used as a source of the receptor protein for the binding assay (Kondo et al. 2008, Ogawa et al. 2008). Since a relatively large amount of receptor-containing membrane fraction is necessary for high-throughput assays, we examined whether a suitable membrane fraction could be prepared from the curd of cauliflower, which is covered in meristematic tissue. The binding assay was performed as previously described (Ogawa et al. 2008), except that 20 μg of cauliflower membrane protein was used for each assay instead of CLV1-ΔKD-HT. The [3H]MCLV3 specifically bound to the cauliflower membrane protein (Fig. 1A). The saturation binding experiment data were subjected to Scatchard analysis (Fig. 1B), which indicated that the $K_d$ value of the cauliflower protein was $1.8 \pm 0.3$ nM. This value is markedly lower than that of CLV1-ΔKD-HT (17.5 nM; Ogawa et al. 2008). Next, a competitive binding assay was performed using 1 nM [3H]MCLV3 and a 100-fold concentration of each alanine-substituted peptide being tested (Fig. 1C). Under these experimental conditions, R1A, G6A, P9A, H11A and H12A did not effectively compete with MCLV3 for binding. The profile was similar to that in the binding assay with CLV1-ΔKD-HT, except that the P9A substitution in that assay resulted in weak interaction (Kondo et al. 2008).

![Figure 1](image-url)

**Fig. 1** Competitive binding assay using cauliflower membrane. (A) Saturation curve of cauliflower membrane and [3H]MCLV3. The diamonds represent the total counts of [3H]MCLV3 co-precipitated with cauliflower membrane. The squares indicate the co-precipitated counts in the presence of excess cold MCLV3 (non-specific). The background-subtracted counts were plotted as the specific counts (triangle). (B) Scatchard plot of [3H]MCLV3. (C) The structure of MCLV3 (top) and the alanine scan of MCLV3 using the competition assay (bottom). NC and PC indicate the non-peptide and positive (MCLV3-added) control, respectively. The amino acid abbreviations at the bottom of C indicate the alanine-substituted analogs at each residue. Each bar represents the tritium count of [3H]MCLV3 specifically bound to the cauliflower membrane protein, normalized to the counts for NC (100%) and PC (0%). All data in B and C represent the average of triplicate experiments. Error bars indicate the SDs.
These data suggest that proline hydroxylation, with its associated decrease in hydrophobicity, is not essential for MCLV3 activity, and that aliphatic modification of the hydroxyl group on the prolines may enable us to develop hydrophobic analogs of MCLV3.

Identification of proline residue properties that are important for bioactivity

A proline residue has a characteristic five-member ring structure (Fig. 3A). In addition, the cis-trans isomerization of proline residues in proteins plays a decisive role in protein folding; this characteristic is attributed to the N-substitution property of imino acids (Reimer et al. 1998). Considering the potential contribution of proline residues to the function of MCLV3, it is important to clarify which properties of the proline residues are essential for their bioactivity. We therefore synthesized analogs in which one or more proline residues were substituted with the simplest imino acid, N-methylglycine (sarcosine, Sar), and evaluated the bioactivity of these analogs (Fig. 3A, B) (Nguyen et al. 1998). A one-way analysis of variance (ANOVA) and Tukey’s tests (α=0.01) revealed that the analog in which Pro4 was substituted with sarcosine (P4Sar) and P7Sar showed significantly lower activity against Arabidopsis roots at 10 nM than did MCLV3, whereas P9Sar was as bioactive as MCLV3. Substitution at Pro4 reduced bioactivity in the multi-substituted analogs (Fig 3B). We next examined the receptor binding activity of these analogs using cauliflower membrane protein (Fig. 3C). P9Sar strongly bound to the cauliflower membrane protein at lower concentrations than did MCLV3, whereas P4Sar and multisubstituted analogs showed weaker binding activities, consistent with the results of the root growth inhibition assay. These data indicate that the imino acid properties of proline, rather than its characteristic five-member ring structure, are important for bioactivity.

Peptoid substitutions within MCLV3

Previously reported methods enabled us to introduce a variety of N-substituted glycines (peptoids) into MCLV3 using commercially available primary amines (Nguyen et al. 1998). We performed a peptoid screening to search for potential analogs by substituting Pro9 of MCLV3 with a series of peptoids. The results of the root growth inhibition assay are shown in Fig. 4. The analogs containing an N-substitution derived from n-butylamine (1), n-hexylamine (2), cyclopropanemethylamine (3), tryptamine (4) or 2-phenylethylamine (5) showed root inhibition activity as strong as that of MCLV3 (Fig. 4A). Amide nitrogen substitution with an isopropyl (6), cyclohexyl (7), cyclohexanemethyl (8) or benzyl (9) group resulted in about one-tenth the activity of MCLV3. The peptoids substituted by (R)- or (S)-1-cyclohexyl (10, 11) or (R)- or (S)-1-phenylethyl (12, 13) groups showed even weaker activity (Fig. 4B). The common property of the weaker analogs is that the substituted groups on the amide nitrogens are bulky, especially at the carbon adjacent to the amide nitrogen. Next, we examined the binding activities of analog 12 (Fig. 5), because it showed the weakest overall activity in the root growth inhibition assay. The binding assay revealed that this weakened analog could still bind to the cauliflower membrane protein, which indicates the possibility of developing CLE analogs that can strongly bind to its receptor without exerting bioactivity. Our results suggest that
this can be accomplished by substituting Pro9 with a suitable bulky peptoid, resulting in a strong antagonist of CLE peptides.

**Discussion**

**Characterization of MCLV3-binding protein from cauliflower**

A protein that can react with an anti-CLV1 antibody is found in the head of cauliflower; this protein is thought to be a CLV1 homolog (Trotochaud et al. 1999). We therefore isolated the membrane fraction from this tissue and examined whether specific binding to MCLV3 could be detected using the [3H] MCLV3 competition assay. We detected dose-dependent specific binding activity in the cauliflower membrane fraction (Fig. 1A). The Scatchard analysis (Fig. 1B) revealed that the $K_d$ value of the binding sites in the cauliflower membrane fraction was 1.8 nM, which was significantly lower than that of CLV1-ΔKD-HT, and was comparable with those of other known receptors of plant peptide hormones (Matsubayashi et al. 2002, 2003).

Fig. 3 (A) The structure of proline and peptoid (R=Me; sarcosine) residues. (B) Root growth inhibition assay of sarcosine-substituted MCLV3 analogs. Each bar represents the average main root length of 20 plants. One-way ANOVA and Tukey’s tests ($\alpha=0.01$) were applied to all the data. Asterisks indicate a significant decrease of the main root length. (C) Competition assay of sarcosine-substituted analogs (upper) and the dose dependence curve of MCLV3 and P9Sar (lower). NC and MCLV3 indicate the non-peptide and positive (MCLV3 added) control, respectively. All data in C represent the average of triplicate experiments. Error bars indicate the SDs.
The density of the binding sites represented by the $B_{\text{max}}$ value was 2.1 pmol mg$^{-1}$ protein, suggesting that the cauliflower membrane includes considerable quantities of the receptor protein that specifically interacts with MCLV3. The availability and preparation convenience of cauliflower provided a useful and high-throughput method for evaluating the receptor binding potential of CLE peptides and analogs.

Effect of proline hydroxylation on MCLV3 activity

The results of the bioassay (Fig. 2) indicate that proline hydroxylation had little influence on MCLV3 bioactivity. One exception is the Pro7,9 hydroxylation which slightly reduced the activity. On the other hand, the full CLV3 peptide is able to bind to the CLV1 ectodomain more tightly

Fig. 4 Root growth inhibition assay of MCLV3 analogs in which Pro9 was substituted by a series of peptoids. (A) Structures and assay results of the analogs that exhibited full activity. (B) Assay results of the analogs in which the bioactivities were reduced by peptoid substitution. Each bar represents the average main root length of 20 plants, and error bars indicate the SDs.

Matsubayashi and Sakagami 2006). The density of the binding sites represented by the $B_{\text{max}}$ value was 2.1 pmol mg$^{-1}$ protein, suggesting that the cauliflower membrane includes considerable quantities of the receptor protein that specifically interacts with MCLV3. The availability and preparation convenience of cauliflower provided a useful and high-throughput method for evaluating the receptor binding potential of CLE peptides and analogs.

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Fig. 5 The dose dependency curve of the binding activities of MCLV3 and peptoid-substituted analog 12. All data represent the average of three experiments. Error bars indicate the SDs.
than can MCLV3, and shows greater activity on Arabidopsis SAMs at low concentrations (Ohyama et al. 2009). These data suggest that proline hydroxylation itself is not important for its conformation and bioactivity, although the hydroxyl group on the Hyp7 residue is necessary as a scaffold for glycosylation.

**Structural features of the peptoids**

The peptoid substitution method is occasionally used to screen for stronger analogs of bioactive peptides of interest (Fowler and Blackwell 2009). Peptoids can mimic the proline residues that are essential for specific protein interactions, and some peptoids can increase the level of interaction (Nguyen et al. 1998). Comparing the configuration of proline residues and peptoids within proteins shows that the ring-free peptoids are more flexible in conformation. Despite this similarity among peptoids, the peptoid analogs of MCLV3 differed in bioactivity, and the bioactivities of the analogs were not highly correlated with the flexibility of the substituted peptoids. The N-substitution, a property common to both prolines and peptoids, also exerts an impact on the configurations of pre-prolyl residues and the amide bonds at the N-terminal side of prolines or peptoids owing to the steric hindrance between the side chain of the pre-prolyl residue and the substituted group on the amide nitrogen (Cheng and Bovey 1977, Reimer et al. 1998). The peptoid screening of Pro9 revealed that the methyl group on the sarcosine residue was enough to enable its bioactivity. On the other hand, an increase in steric bulkiness of the substitutions at the amide nitrogen, which is expected to severely restrict the configuration of the pre-prolyl residue, reduced bioactivity. In addition, an MCLV3 analog in which glycine substitution at Asp8 reduced the steric hindrance between Asp8 and the substituted group on the amide nitrogen (data not shown). These data suggest that the moderate restriction of Asp8 configuration provided by Pro9 is a prerequisite for MCLV3 activity.

Alanine scan analyses and swap experiments between MCLV3 and TDIF (Kondo et al. 2008) indicated that the Arg1, His11 and His12 residues of MCLV3 are probably recognized by the receptor CLV1, whereas the role of Pro9, which is highly conserved in CLE peptides and necessary for bioactivity but not essential for receptor binding, was previously unclear. Our SAR studies indicate that the Pro9 residue plays an important role in maintaining the bioactive conformation of the peptide chain. In addition, the restricted flexibility of Asp8 provided by N-substitution of Pro9 can account for the results of the SAR analyses. The use of Pro9 substitutions to maintain Asp8 and Pro9 in a conformation suitable for receptor binding and to suppress Asp8 rotation is a strategy that can be applied to developing potential CLE peptide antagonists. Future study should focus on the in vivo application of synthesized potential antagonists, and now we are seeking a suitable and highly sensitive bioassay for them.

**Materials and Methods**

**General procedures**

We used an Esquer 3000 ESI-IT system (Applied Biosystems) equipped with an Agilent 1100 Series HPLC system (Agilent Technologies) for electrospray ionization ion-trap mass spectrometry (ESI-IT MS). A 4700 Proteomics Analyzer (Applied Biosystems) was used for matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) and tandem MS (MS/MS) using α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix.

**Peptide synthesis and purification**

All peptides were synthesized by Fmoc (9-fluorenylmethoxy-carbonyl) solid-phase peptide synthesis using a peptide synthesizer (ABI model 433A). The O-methylhydroxyproline-substituted peptides were synthesized using Fmoc-O-methyl-Hyp (14). Boc-Hyp-OBzl (Watanabe Chemicals) was methylated using iodomethane in the presence of Ag2O. The Boc (tert-butoxy carbonyl) group was deprotected by 50% trifluoroacetic acid (TFA) in dichloromethane, then the consequent imine was protected by Fmoc using Fmoc-succinimide in 1N NaHCO3/aqueous methanol. Finally, the benzyl group was deprotected by H2-Pdi/C to obtain compound 14. For compound 14 [Fmoc-Hyp(Me)-OH], the nuclear magnetic resonance (NMR) and MS measurements were as follows: 1-H-NMR (400 MHz, CDCl3, δH, 2.08–2.17 (0.25 H, m), 2.31–2.45 (1.75 H, m), 3.32 (3 H, s), 3.48–3.61 (1 H, m), 3.64–3.78 (1 H, m), 4.16 (0.3 H, t, J=6.9 Hz), 4.28 (0.7 H, t, J=6.9 Hz) 4.34–4.55 (3 H, m), 7.28–7.45 (4 H, m), 7.53–7.62 (2 H, m), 7.71–7.81 (2 H, m), HRMS m/z [M+H]+: expected value for C17H22NO5, 368.1493; actual, 368.1493. The sarcosine-substituted peptides were synthesized from Fmoc-sarcosine (Watanabe Chemicals). Peptoid substitution was performed as previously described (Nguyen et al. 1998). Synthesized peptides were deprotected and cleaved from the resin with TFA cleavage cocktail with 2-mercaptoethanol. The peptides were purified by reverse-phase HPLC comprising two solvent delivery pumps and a UV detector set at 220 nm. The column used was a Develosil-OBS HG-5, 10 mm i.d.×250 mm, coupled to a guard column (Nomura Chemical Co.). The separation was carried out with a linear gradient of solvent A (H2O/CH3CN 98:2 + 0.5% acetic acid) into solvent B (H2O/CH3CN 30:70 + 0.5% acetic acid); 0–50% B over 20 min, 50–100% B over 5 min, run at 3.0 ml min⁻¹. The purity of each peptide was assessed by its UV spectrum (210 nm) and total ion chromatogram of liquid chromatography (LC)-MS analysis.

**Bioassay and receptor binding assay**

The root growth inhibition assay was performed as described previously (Kondo et al. 2006). The cauliflower heads (Brassica oleracea var. botrytis, produced in Nagano prefecture, Japan) were purchased in a market. Epidermal fragments (about 1 mm thick) of the cauliflower head were collected and ground with a pestle and mortar, then suspended in 40 ml of binding
buffer (20 mM MES-KOH, pH 5.5, 200 mM sucrose). The suspension was centrifuged at 2,580 $\times$ g (4°C for 10 min) to remove the cell debris. The supernatant was centrifuged at 71,680 $\times$ g for 30 min, and the resulting pellets were used as the membrane protein fraction. We synthesized and purified tritiated MCLV3 (73.3 mCi mmol$^{-1}$ for the experiment in Fig. 1, and 10.5 mCi mmol$^{-1}$ for the other experiments), then performed the receptor binding assay as described by Ogawa et al. (2008).

**Funding**

This research was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) of Japan; the Ministry of Education, Culture, Sports, Science and Technology of Japan [Grants-in-Aid for Scientific Research (S) (18101009)].

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