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Original Paper

CLE14 peptide signaling in Arabidopsis root hair cell fate determination

Naoto Hayashi[1], Takuya Tetsumura[2], Shinichiro Sawa[3], Takuji Wada[1], Rumi Tominaga-Wada[1,*]

[1] Graduate School of Biosphere Sciences, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima 739-8528, Japan; [2] Department of Agricultural and Environmental Sciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-Nishi, Miyazaki 889-2192, Japan; [3] Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan
* E-mail: rtomi@hiroshima-u.ac.jp Tel & Fax: +81-82-424-7966

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Abstract Morphological adjustment is a critical strategy for the survival of plant species in various environments. The CLE (CLAVATA3/EMBRYO SURROUNDING REGION) family of plant polypeptides is known to play important roles in various physiological and developmental processes and the relevant signaling pathways are conserved in diverse land plants. Previously, it has been suggested that overexpression of *CLE14* promotes root hair cell differentiation in Arabidopsis roots. To clarify this suggested function of CLE14 peptide on root hair induction, we examined the effect of synthetic CLE14 peptide on Arabidopsis root hair development. Consistent with the results of previous overexpression analyses of *CLE14*, we demonstrated that application of synthetic CLE14 peptide induced excess root hair formation on CLE14-treated Arabidopsis roots. In addition, CLE14 reduced the expression of the non-hair cell fate determinant gene, *GLABRA2*. Our results thus indicate that CLE14 can activate the transcriptional regulatory cascade of root hair formation.

Key words: Arabidopsis, CLE14, *CPC*, *GL2*, root hair.

Introduction

Plant morphological plasticity is an important strategy for survival under adverse environmental conditions. Peptide hormones, which are secreted as signaling molecules, are important for intercellular communication in multicellular organisms. In Arabidopsis, *CLAVATA3* (*CLV3*) was the first characterized *CLV3*/*EMBRYO SURROUNDING REGION* (*CLE*) gene and was shown to encode a peptide hormone that controls the number of stem cells in the shoot apical meristem (SAM) (Fletcher et al. 1999). Mutations in the *CLV3* gene affect the size of the SAM (Clark et al. 1995). Arabidopsis harbors 32 *CLE* genes (Betsuyaku et al. 2011). These are assumed to act as 12- to 13-amino acid peptide hormones that regulate cellular activity in the SAM and root apical meristem (RAM), as well as in vascular tissues (Cock and McCormick 2001; Ito et al. 2006; Kondo et al. 2006; Ohyama et al. 2008, 2009). Genes homologous to *CLE* have also been identified in various other plant species (Han et al. 2016; Miwa et al. 2009; Oelkers et al. 2008; Tominaga-Wada et al. 2013). Previously, one of the Arabidopsis *CLE* genes, *CLE14*, has been reported to be expressed in the root tip, including root epidermis and root hairs (Meng and Feldman 2010). Overexpression of the *CLE14* gene has been shown to trigger early differentiation of root epidermal cells, leading to root hair development (Meng and Feldman 2010).

Root hairs play important roles in plant growth and development via their water absorption, nutrient uptake, and anchorage functions. In growing Arabidopsis roots, epidermal cells differentiate into two types of cells, root hair cells and non-hair cells, in a file-specific manner. Epidermal cells that are in contact with two underlying cortical cells differentiate into root hair cells, whereas the cells in contact with only one cortical cell differentiate into non-hair cells (Berger et al. 1998; Dolan et al. 1993, 1994; Galway et al. 1994). Several transcription factors involved in root hair or non-hair cell fate determination have been identified. The *GLABRA2* (*GL2*) gene encodes a homeodomain leucine-zipper protein (Rerie et al. 1994), whereas the *WEREWOLF* (*WER*) gene encodes an R2R3-type MYB transcription factor (Lee and Schiefelbein 1999), and the *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*) genes encode basic helix-loop-helix (bHLH) transcription factors (Bernhardt et al. 2003). A transcriptional protein

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complex, including WER and GL3/EGL3, acts upstream of the *GL2* gene and promotes *GL2* gene expression, leading to non-hair cell differentiation (Bernhardt et al. 2003, 2005; Galway et al. 1994; Hung et al. 1998; Lee and Schiefelbein 1999; Rerie et al. 1994; Wada et al. 1997). In contrast, root hair cell differentiation is controlled by the *CAPRICE* (*CPC*) gene, which encodes an R3-type MYB transcription factor (Wada et al. 1997). The CPC protein disturbs the formation of the GL3/ETC3-WER transcriptional complex by competitively binding with WER, and represses the expression of *GL2*, thereby inducing root hair cell differentiation (Koshino-Kimura et al. 2005; Kurata et al. 2005; Tominaga et al. 2007; Wada et al. 2002). Therefore, the *GL2* gene is a decisive factor, acting farthest downstream in this root hair/non-hair cell regulatory cascade.

In this study, we sought to elucidate the relationship between CLE14 peptide activity and the transcriptional cascade of the root hair/non-hair differentiation system. To clarify the effect of CLE14 peptide application on root hair development, we analyzed the expression of the *GL2* and *CPC* genes. On the basis of our observations, we propose a model of root hair formation showing transcriptional regulation by CLE14 peptide. Recently, the importance of the CLE14 signaling system in the phosphate starvation response was demonstrated (Gutierrez-Alanis et al. 2017). CLE14 application may have a valuable role in agriculture as it could be used to induce the formation of additional root hairs in crop species and allow the use of lower amounts of phosphorus fertilizers for growing crops.

Materials and methods

Plant material and growth conditions

In this study, we used the *Arabidopsis thaliana* (L.) Heynh. Col-0 ecotype as the wild type. The seeds were surface-sterilized and sown on the surface of 1.5% agar plates as described previously (Okada and Shimura 1990). Plates containing the seeds were maintained at 4°C for 2 day and then incubated at 22°C under constant white light (50–100 μmol m^{-2} s^{-1}).

Synthetic peptides

Synthetic CLE14 peptides were obtained from Eurofins Genomics (Tokyo, Japan) and were used at 73.25% purity. The CLE14 peptides were dissolved in 0.1% trifluoroacetic acid (TFA) to produce a 1 mM stock solution that was sequentially diluted to working concentrations prior to use. Mock treatment was performed using the same volume of 0.1% TFA. Stock and working solutions were stored at −20°C.

Real-time RT-PCR

Total RNA was extracted from the roots of seedlings using an RNeasy plant mini kit (Qiagen). On-column DNase I digestion was performed during RNA purification following the protocol described in the RNeasy Mini Kit handbook. First-strand cDNA was synthesized from 200 ng of total RNA in a 10-μl reaction mixture using the PrimeScript RT Master Mix (Perfect Realtime) (TaKaRa). Real-time PCR was performed using the StepOne Real-Time PCR System (Applied Biosystems) with Fast SYBR Green Master Mix (Applied Biosystems). Real-time PCR was used to analyze the mRNA levels of the transcripts encoding *PDF2*, *GL2*, and *CPC*. The relative expression of each transcript was calculated using the ΔΔCt method (Livak and Schmittgen 2001). *PDF2* was used as an endogenous control for normalization of the expression levels of *GL2* and *CPC*. The primers used were as follows: PDF2-F (TAA CGT GGC CAA AAT GAT GC) and PDF2-R (GTT CTC CAC AAC CGC TTG GT) for *PDF2*, GL2-F (TCG GAT CAC TGA GAC CAC AA) and GL2-R (GTG TAT CCC GGA ACC AGT GT) for *GL2*, and CPC-F (GGA TGT ATA AAC TCG TTG GCG ACA G) and CPC-R (GCC GTG TTT CAT AAG CCA ATA TCT C) for *CPC*.

Histology

Primary roots of 10-day-old *GL2p:GUS* transgenic plants were excised from the growth medium with or without CLE14 peptide (0.5 μM), and were stained in GUS staining solution (0.05 M sodium phosphate buffer, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide). Excised primary roots were incubated at 37°C for 30 min.

Microscopy

For observation of the roots, specimens were examined by light microscopy using a Leica MZ16FA stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were recorded using a high-sensitivity CCD color camera system (VB 7010; Keyence, Osaka, Japan). For observation of GUS-stained roots, we used a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany). Images were recorded using an AxioCam camera (Carl Zeiss).

Results and discussion

Effect of synthetic CLE14 peptide on root hair formation

It has previously been suggested that overexpression of the *CLE14* gene induces early differentiation of root hairs on Arabidopsis root tips (Meng et al. 2010). Given that root hair proliferation may increase water and nutrient uptake by roots from the soil, leading to higher crop yields, we considered that increasing the number of root hairs by treatment with CLE14 peptide might have valuable agricultural applications. However, whether the many-root hair phenotype is maintained during root maturation has not been clarified (Meng et al. 2010). In addition, for agricultural use, it is necessary to confirm the activity of exogenous CLE14 administration (as opposed to endogenous overexpression) on root hair

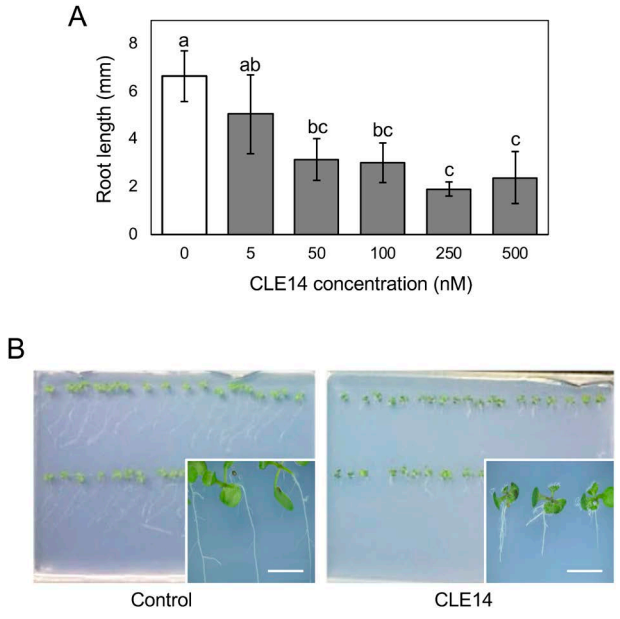

Figure 1. Root phenotypes of CLE14-treated Arabidopsis seedlings. (A) Root lengths of Arabidopsis seedlings grown under different CLE14 concentrations. Data are means±SD (n=5). The different letters indicate statistically significant differences according to ANOVA and post-hoc Bonferroni test (p<0.01). (B) Phenotypes of CLE14-treated Arabidopsis seedlings. Seven-day-old Arabidopsis seedlings grown in control medium (1/2MS; left) or in 1/2MS medium containing 0.5 μM of the CLE14 peptide (right). Scale bars: 1 mm.

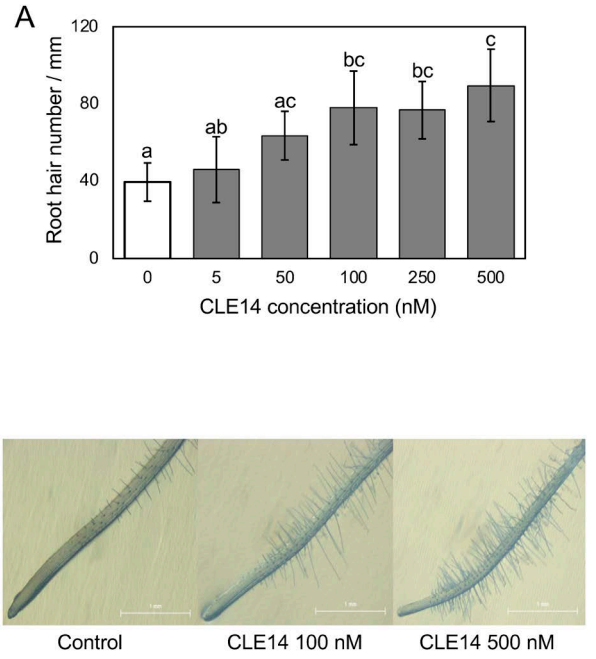

Figure 2. Root hair phenotypes of CLE14-treated Arabidopsis seedlings. (A) Root hair numbers of Arabidopsis seedlings grown under different CLE14 concentrations. Data are means±SD (n=5). The different letters indicate statistically significant differences according to ANOVA and post-hoc Bonferroni test (p<0.05). (B) Root hair phenotypes of two-week-old Arabidopsis seedlings grown in control medium (1/2MS; left), in 1/2MS medium containing 100 nM of CLE14 peptide (middle), or in 1/2MS medium containing 500 nM of CLE14 peptide (right). Scale bars: 1 mm.

formation. Therefore, we examined the effect of synthetic CLE14 peptide application on Arabidopsis roots. As previously reported, the primary root growth of seedlings was inhibited by exogenous application of synthetic 12-amino acid CLE14, as characterized by a marked reduction in primary root lengths (Figure 1) (Meng and Feldman 2010). As CLE peptides are reported to cause a decrease in the number of root meristematic cells (Fiers et al. 2005), the reduction in root length might be due to a lower rate of cell division. As the concentration of CLE14 increased from 0 to 500 nM, the root length was significantly reduced (Figure 1A). This dwarf root phenotype is similar to that exhibited by plants with *CLE14* overexpression (Figure 1B) (Meng et al. 2010).

Exogenous application of synthetic CLE14 induced root hair formation (Figure 2). We observed a significant increase in root hair number as the concentration of CLE14 increased from 0 to 500 nM (Figure 2A). The CLE14 peptide-treated two-week-old seedlings showed a marked increase in the number of root hairs compared with that in control plants (Figure 2B). These results suggest that the many-root hair phenotype induced by CLE14 peptide is maintained during root maturation.

CLE14 inhibits GL2 gene expression

To investigate the effect of CLE14 peptide on genes regulating root hair cell fate determination, we performed real-time PCR analyses. We detected a significantly lower accumulation of *GL2* transcripts in the CLE14 peptide-treated Arabidopsis roots compared with that in control plants (Figure 3A). *GL2* gene expression levels decreased to a quarter of that in the controls (Figure 3A). This result suggests that the CLE14 peptide affects the gene transcriptional control pathway that is involved in root hair cell fate determination-related. The *GL2* gene is believed to be the farthest downstream acting gene in the transcriptional regulatory cascade for root epidermal cell fate determination (Tominaga-Wada et al. 2011). Therefore, a reduction in *GL2* gene expression implies the potential to enhance root hair cell formation. We further analyzed the expression levels of the *CPC* gene, which encodes an R3-type MYB transcription factor, and induces root hair formation (Wada et al. 1997). In contrast to *GL2* gene expression, the level of *CPC* expression was significantly higher in the CLE14 peptide-treated roots than in the roots of control plants (Figure 3B). This result suggests that the CLE14 peptide is involved in the CPC-GL2-related root hair forming pathway. To confirm the effect of CLE14 peptide on *GL2* gene expression, we performed a dose-dependent response analysis. As the concentration of CLE14 increased from 0 to 500 nM, the expression levels of *GL2* decreased (Figure 3C). These results suggest that CLE14 peptide affects the CPC-GL2-related root hair

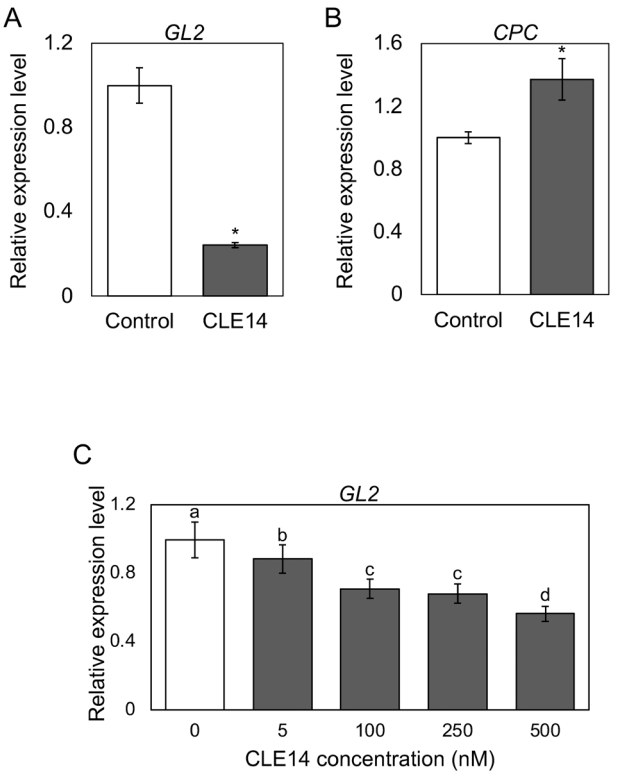

Figure 3. Expression analysis of the *GL2* and *CPC* genes in Arabidopsis roots treated with or without CLE14 peptide. (A) Real-time reverse transcription PCR analysis of *GL2* gene expression in the roots of 10-day-old seedlings grown in control medium (1/2MS) or in 1/2MS medium containing 0.5 μM of CLE14 peptide. (B) Real-time reverse transcription PCR analysis of *CPC* gene expression in the roots of 10-day-old seedlings grown in control medium (1/2MS) or in 1/2MS medium containing 0.5 μM of CLE14 peptide. Expression levels of *GL2* and *CPC* in each sample relative to those in control plants are shown. Experiments were repeated three times. Error bars indicate the standard error. Bars marked with asterisks indicate a significant difference between control and CLE14-treated plants as determined by Student's *t*-test ($p<0.05$). (C) Real-time reverse transcription PCR analysis of *GL2* gene expression in the roots of 10-day-old seedlings grown under different CLE14 concentrations. Expression levels of *GL2* in each sample relative to those in control (0 nM) plants are shown. Experiments were repeated eight times. Data are means±SD ($n=80$). The different letters indicate statistically significant differences according to ANOVA and post-hoc Bonferroni test ($p<0.01$).

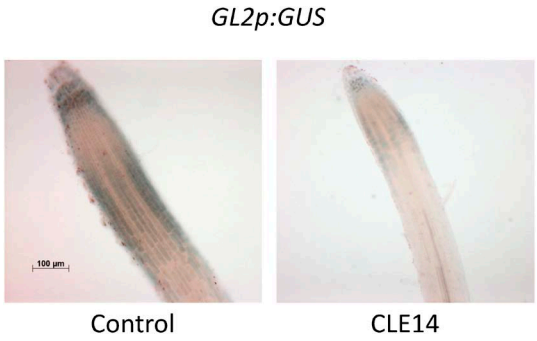

Figure 4. Histochemical staining of GUS activity in *GL2p:GUS* transgenic Arabidopsis plants. Expression of the *GL2p:GUS* reporter in the roots of 7-day-old seedlings grown in control medium (1/2MS) or in 1/2MS medium containing 0.5 μM of CLE14 peptide and stained with X-Gluc. Scale bar: 100 μm.

formation pathway.

GL2p:GUS gene expression is normally observed in root epidermal cells, mainly in the non-hair cell files in Arabidopsis roots (Figure 4) (Masucci et al. 1996). To define the effect of CLE14 peptide on tissue-specific *GL2* expression patterns, we treated *GL2p:GUS* transgenic plants with CLE14 peptide. Consistent with the result of real-time PCR analysis (Figure 3A, 3C), *GL2p:GUS* gene expression was markedly repressed by the CLE14 peptide treatment (Figure 4). These results strongly suggest that the CLE14 peptide can inhibit *GL2* expression. Collectively, our observations indicate that the CLE14 peptide has the latent potential to regulate gene expression during root hair formation.

CLE peptides are known to negatively regulate stem cell fates in the shoot apical meristem (SAM) and root apical meristem (RAM) in Arabidopsis (Meng et al. 2010). However, in contrast to most CLE peptides, CLE40 has been reported to promote differentiation of stem cells in the RAM of Arabidopsis by restricting *WOX5* expression (Stahl et al. 2009). Previously, overexpression of *CLE14* has been reported to induce both short-root phenotypes and early root hair differentiation (Meng et al. 2010). These observations imply that CLE14 peptide may have two different functions in roots: one is the inhibition of primary root growth, whereas the other is the induction of root hair differentiation.

In this study, we focused on the effect of synthetic CLE14 peptide application on root hair differentiation and analyzed the relationship between the CLE14 peptide hormone and root hair/non-hair cell fate determination-related transcription factors. We demonstrated that synthetic CLE14 peptide also increases the number of root hairs on Arabidopsis mature root epidermis (Figure 2). In addition, *GL2* gene expression was strongly inhibited, and *CPC* gene expression was significantly enhanced by CLE14 treatment (Figure 3). Therefore, the CLE14 peptide hormone is evidently involved in the CPC-GL2 transcriptional regulatory cascade of root hair/non-hair cell differentiation. GL2 is known to inhibit root hair formation, and CPC is known to inhibit *GL2* gene expression to control root hair/non-hair cell differentiation (Figure 5A) (Tominaga-Wada et al. 2011). As most CLE peptides work by interacting with receptor-like kinases (RLKs), CLE14 should also act via an RLK (Gutierrez-Alanis et al. 2017). Again, our results show that the CLE14 peptide inhibited *GL2* gene expression and enhanced *CPC* gene expression, likely via an RLK, suggesting that this peptide hormone contributes to root hair induction (Figure 5B). Recently, phosphate starvation was reported to induce *CLE14* expression to trigger root meristem differentiation (Gutierrez-Alanis et

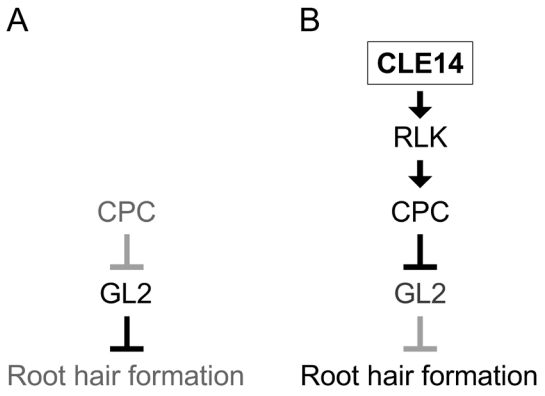

Figure 5. Model of root hair formation showing the regulation and proposed role of CLE14 peptide. (A) Root hair formation is inhibited by GL2. *GL2* gene expression can be reduced by CPC. (B) CLE14 peptide induces *CPC* gene expression via RLK and inhibits *GL2* gene expression. As a result, CLE14 induces root hair formation.

al. 2017), and Pi deficiency is known to induce root hair differentiation (Salazar-Henao et al. 2016). Our results may connect these two processes and implicate other molecular players in CLE14 peptide signaling, including GL2 and CPC (Figure 5B). We further confirmed the reduction in *GL2* gene expression by CLE14 using *GL2p:GUS* transgenic plants (Figure 4). Previously, overexpression of the *CLE14* gene in Arabidopsis has been reported to induce a significant reduction in leaf trichome number (Meng and Feldman 2011). The trichome-less and many-root hair phenotype is similar to that of the *gl2* mutant (Tominaga-Wada et al. 2011). If the CLE14 peptide inhibits *GL2* gene function in the whole plant body in *35S:CLE14* transgenic plants, it may result in a phenotype similar to that of the *gl2* mutant. Therefore, it would be reasonable to assume that the CLE14 peptide triggers root hair formation in the CLE14-treated plants. In this study, we showed that the CLE peptide treatment induced root hair development (Figure 2). Although further investigations of the effect of CLE14 peptide on root hair formation will be required, the present study does provide new insights into the molecular basis of CLE peptide signaling in Arabidopsis root hair/non-hair cell differentiation.

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