Long-distance translocation of CLAVATA3/ESR-related 2 peptide and its positive effect on roots sucrose status

Satoru Okamoto,1,2,* Azusa Kawasaki,1 Yumiko Makino,3 Takashi Ishida4 and Shinichiro Sawa5,†

1 Graduate School of Science and Technology, Niigata University, Niigata, 950-2181, Japan
2 PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan
3 National Institute for Basic Biology, Okazaki, Aichi 444-8585, Japan
4 International Research Organization for Advanced Science and Technology (IROAST), Kumamoto University, Kumamoto, 860-8555, Japan
5 Graduate School of Science and Technology, Kumamoto University, Kumamoto, 860-8555, Japan

*Author for correspondence: okamoto@agr.niigata-u.ac.jp
†Senior author

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Abstract

In vascular plants, roots anchor themselves into the soil and take up water and nutrients to provide them to the shoots. Therefore, continuous growth and development of the roots are important for plant life. To achieve this, photosynthesizing leaves must be able to supply sufficient photoassimilates to the roots. However, the mechanisms by which plants maintain carbon levels in roots remain elusive. Here, we focused on the Arabidopsis (Arabidopsis thaliana) CLAVATA3/ESR-related 2 (CLE2) peptide, which was detected in Arabidopsis xylem exudate, and its homologs. CLE2 and CLE3 genes responded to carbon-deficient conditions. Loss- and gain-of-function mutant analyses showed that CLE genes positively affected root sucrose level. Mutations in the CLE genes resulted in a high shoot/root ratio under sucrose-free conditions. Grafting experiments demonstrated the systemic effect of CLE peptide genes. These findings provide insights into the molecular basis for the relationship between roots and leaves in maintenance of the root sucrose levels and growth.

Introduction

Plants fix CO2 into carbohydrates through photosynthesis, and in many plant species, sucrose is the major photoassimilate that is exported from photosynthesizing leaves (source) to nonphotosynthesizing (sink) organs. In the leaves, sucrose is transported symplastically from mesophyll cells to phloem parenchyma cells (Giaquinta, 1983). In apoplastic loading plants, including Arabidopsis (Arabidopsis thaliana), sucrose is exported by SWEET efflux transporters from parenchyma cells to the apoplast (Chen et al., 2012) and then loaded into the sieve element–companion cell complex by an energy-dependent sucrose symporter called sucrose–proton symporter 2 (SUC2) in Arabidopsis (Truernit and Sauer, 1995; Stadler and Sauer, 1996; Gottwald et al., 2000). It has been reported that sucrose transporter activity is regulated transcriptionally and posttranscriptionally (Vaughn et al., 2002; Fan et al., 2009; Xu et al., 2018, 2020), which would support plasticity in the phloem loading of sucrose. In roots,
which are a major sink organ, sucrose is unloaded from phloem and is consumed via respiration associated with growth and ion uptake. In addition, a non-negligible amount of carbon is secreted to the rhizosphere. Some of this carbon contributes to the establishment of plant microbe interactions or to the acquisition of micronutrients (Jones et al., 2009).

Roots play important roles during plant life; that is, they anchor the plant body into the soil, take up water and inorganic nutrients, and establish interactions with soil microbes (Lincoln and Eduardo, 2018). Therefore, maintaining proper root growth and development is essential for plants to survive under various environmental conditions. To achieve this, the leaves must translocate sufficient amounts of photoassimilates to the roots. Meanwhile, the amounts of photoassimilates that plants acquire throughout the day are limited, and in the field, plants also experience diurnally fluctuating light conditions. To ensure efficient management of the carbohydrate supply to the sink roots, the source leaves need information regarding the carbon requirements of the roots. To date, many studies have addressed the sucrose metabolism pathways in photosynthesizing source organs such as leaves and nonphotosynthesizing sink organs such as roots (Stitt and Zeeman, 2012; Ruan, 2014; Rossi et al., 2015), as well as the mechanism of how sucrose is loaded into the phloem for long-distance transport (Gottwald et al., 2000; Chen et al., 2012). Moreover, various environmental factors, including nutrient availability, have been reported to affect carbon allocation in plants (Hermans et al., 2006; Lemoine et al., 2013). At the cellular level, Snf1-related protein kinase 1 and target-of-rapamycin (TOR) are known to function in sensing and signaling for energy status (Menand et al., 2002; Baena-González et al., 2007; Robaglia et al., 2012). They also integrate various stimuli, such as energy, nutrient, and environmental stress, and control cell growth, metabolism, and proliferation. However, it remains elusive how the sink and source organs communicate with each other to control carbon status.

CLAVATA3/ESR-related (CLE) is a well-known plant peptide family and plays divergent roles in the plant life cycle (Yamaguchi et al., 2016; Fletcher, 2020). Bioactive mature CLE peptides consist of 12 or 13 amino acids derived from the conserved C-terminal region of their precursor polypeptides (Ito et al., 2006; Kondo et al., 2006; Ohyama et al., 2009; Okamoto et al., 2013, 2015; Takahashi et al., 2018). It has been shown that a few CLE peptides are involved in root-to-shoot signaling, such as the regulation of nodule formation and drought tolerance (Okamoto et al., 2013; Takahashi et al., 2018). Among Arabidopsis CLEs, the amino acid sequence of putative CLE1–7 peptides is highly conserved, and according to Zhang et al. (2020), CLE1–7 are classified into Group 1A. To date, it has been reported that CLE1–7 genes respond to various external or internal stimuli (Araya et al., 2014; Dong et al., 2019; Ma et al., 2020).

In this study, we focused on Arabidopsis CLE2 and its homologs and characterized their function. Mass spectrometric analysis showed that the mature CLE2 peptide is present in Arabidopsis xylem exudate. Quantitative expression analysis demonstrated that the mRNA level of CLE2 was upregulated under low-carbon conditions. Loss- and gain-of-function analyses of CLE2 and its homologs revealed that the CLE genes positively regulate the root sucrose level. Furthermore, mutations in the CLE genes led to a high shoot/root ratio, and this phenotype was restored by the application of sucrose to the media.RAFT experiments demonstrated that the root genotype of the CLE genes systemically affects the mRNA level of SUC2 in leaves. Our results provide insights into the relationship between long-distance mobile CLE peptides and maintenance of the sucrose status and growth of roots.

Results

CLE2 peptide is present in xylem exudate

Through a peptidomics analysis of soybean (Glycine max) xylem exudate, we previously identified multiple root-to-shoot long-distance mobile peptides (Okamoto et al., 2015). Among the peptides identified, xylem sap-associated peptide 4 (XAP4, also reported as GmCLE32; Hastwell et al., 2015) was mainly expressed in the roots, and recently, we found that XAP4/GmCLE32 responds to low-carbon conditions (Supplemental Figure S1). The expression of XAP4/GmCLE32 in roots was strongly upregulated by shoot excision, defoliation, and continuous dark conditions but did not respond to decapitation. Furthermore, the application of sucrose inhibited the response, but mannose did not.

To explore the function of XAP4/GmCLE32, we focused on homologs in Arabidopsis CLEs. The amino acid sequence of the XAP4/GmCLE32 peptide shows high similarity to that of Arabidopsis CLE1–7 (Supplemental Figure S2). To explore whether the Arabidopsis CLE peptide is translocated from roots to shoots via the xylem, we collected Arabidopsis xylem exudate and performed nano-liquid chromatography-tandem mass spectrometry (nano-LC–MS/MS) analysis (Figure 1A). Previously, mature CLE2 peptide was identified as a 12-amino acid glycopeptide, in which the seventh hydroxyproline residue is modified with three arabinose residues (Ohyama et al., 2009). We indeed detected a peak of m/z 863.4 at 9.0 min that corresponded to this mature CLE2 peptide from Arabidopsis xylem exudate (Figure 1B). The MS/MS spectrum of this peak showed a fragmentation pattern coinciding with that of the 12-amino acid arabinosylated CLE2 peptide (Figure 1C). This result supports the root-to-shoot translocation of Arabidopsis CLE peptides.

To examine whether CLE2 responds to low-carbon conditions, expression analysis of CLE2 was performed on plants treated with the photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or prolonged darkness. CLE2 was primarily expressed in roots, and CLE2 mRNA levels were upregulated by DCMU and dark treatments (Figure 1D). On the other hand, CLE2 upregulation was diminished by sucrose application to the media. In addition, we examined the expression of CLE1 and CLE3–7 and found that CLE3 was also upregulated under low-carbon conditions (Supplemental Figure S3), while CLE4 and CLE7 were downregulated. Recently, Ma et al. (2020) reported that CLE2 and
CLE3 respond to dark treatment, and we further clarified that this response was inhibited by the application of sucrose to roots. Thus, CLE2 and CLE3 are likely upregulated by low-carbon availability conditions in the roots. CLEs positively regulate the sucrose contents in roots

Considering that the mRNA level of CLE2 was upregulated under low-carbon conditions (Figure 1D), we assumed that CLE2 is involved in signaling related to carbon source availability. To characterize the functions of CLE2, sugar (sucrose and glucose) and starch contents in the roots and leaves were measured. We sampled plants at the end of the day in the absence of stress, since differences in carbon contents are difficult to detect under extreme energy stresses. First, we examined cle2 phenotypes, but no significant differences were observed in sugar and starch contents in its roots and leaves (Supplemental Figure S4A). Because the mRNA level of CLE3 was also upregulated under low-carbon conditions, we examined the phenotype of the cle3 mutant, but the same result was obtained. The amino acid sequences of putative mature CLE1–7 peptides are highly conserved (Supplemental Figure S2B). Therefore, these peptides likely share overlapping biological activities that are difficult to deduce with single mutants. To fully analyze the functions, we then generated a cle2cle3 double mutant and a cle1-7 septuple mutant using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology (Yamaguchi et al., 2017; Supplemental Figure S5). As a result, in roots, cle2cle3 showed sucrose and glucose levels similar to those of wild-type (WT) plants. In contrast, the cle1-7 septuple mutant roots exhibited a significant decrease in sucrose content (Supplemental Figure S4A). In mature leaves, sucrose levels diminished not only in cle1-7 but also in cle2cle3. These results suggest that CLEs affect sucrose levels in roots and leaves in an independent manner.

To further characterize the effect of CLEs, we conducted a gain-of-function analysis of CLE2. It has been shown that the promoter activities of CLE1–7 are detected in steles (Jun et al., 2010), and the spatial pattern of CLE2 and CLE3 promoter activity is not altered by dark treatment (Ma et al., 2020); therefore, we used the Commelina Yellow Mottle Virus (CoYMV) promoter, which is known to be active in vascular tissues (Medberry et al., 1992). We confirmed that the mRNA level of CLE2 in the transgenic lines was higher than that in the WT line (Supplemental Figure S6). In roots, ectopic

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**Figure 1** Detection of the CLE2 peptide from Arabidopsis xylem exudate. A, Arabidopsis xylem exudate was collected from cut surfaces of hypocotyls. B, Base peak chromatogram of purified Arabidopsis xylem exudate. C, The MS/MS spectrum of m/z 863.4 eluted at 9.0 min in (B) showed a fragmentation pattern identical to that of arabinosylated CLE2 peptides. B and C, One typical experiment of two replicates is shown. D, Relative mRNA level of the CLE2 gene. Plants grown on sucrose-free or 1% sucrose-containing media were treated with 50-μM DCMU or incubated in darkness for 24 h. The expression levels of each CLE2 gene in the treated samples were normalized to control roots. The dots represent individual measurements. Each result is the mean ± standard error of the mean (SEM) of measurements obtained from three independent experiments.
expression of CLE2 led to significantly higher sucrose levels than those in WT plants (Figure 2). On the other hand, the sucrose contents in mature leaves remain unchanged. In addition, we examined the phenotype of pCoYMV:CLE3 lines and found that the root sucrose content was also upregulated in these lines (Supplemental Figure S4B).

Furthermore, the synthetic mature CLE2 peptide was transiently applied to Arabidopsis (Supplemental Figure S7). Our mass spectrometric analysis suggests that the CLE2 peptide is translocated from roots to shoots (Figure 1, A–C), so the CLE2 peptide was applied to the rosette leaves of WT plants. Twenty-four hours after the application, the starch and sugar contents in shoots and roots were measured. As observed in the pCoYMV:CLE2 lines, the plants treated with the CLE2 peptide showed significantly higher sucrose levels in roots. We further applied the CLE2 peptide to the cle1-7 septuple mutant and found that the sucrose contents were restored by the application (Supplemental Figure S8). Taken together, we conclude that CLEs positively regulate the sucrose level in roots.

**Sucrose rescues root growth in the cle1-7 mutant**

In the cle1-7 mutant, the root sucrose level was lower than that in the WT plants (Supplemental Figure S4A). The growth and development of organs require additional energy and resources for de novo synthesis of cellular components. Considering that sucrose is the major carbon source in sink organs, there is a possibility that the decrease in cle1-7 root sucrose levels affects root growth. To explore this possibility, we evaluated shoot and root mass in the cle1-7 mutant. Under normal light (constant light intensity) conditions, the cle1-7 shoot/root mass ratio was ~20% higher than that of WT plants, and the total root mass was slightly decreased on sucrose-free media (Figure 3, A and C). These defects were completely restored by the application of sucrose to the media. On the other hand, under our growth conditions, no significant differences in shoot mass were found between the cle1-7 septuple mutant and the WT plants, regardless of sugar availability. Furthermore, no significant differences were observed in cle2, cle3, and cle2cle3 mutants compared with WT plants. Thus, sucrose is required for maintenance of the shoot/root ratio in the cle1-7 septuple mutant.

It has been reported that the phenotypes of mutants that are involved in photosynthesis or sugar metabolism are enhanced under temporally heterogeneous light conditions (Bellafiore et al., 2005; McCormick and Kruger, 2015). In the field, light conditions vary diurnally; thus, we examined the growth of cle mutants under diurnally fluctuating light conditions. As a result, the shoot/root mass ratio in the cle1-7 septuple mutant was ~40% higher than that in WT plants (Figure 3, B and D). This phenotype was enhanced compared with that under normal light conditions and restored by the application of sucrose to the media. Under diurnally fluctuating light conditions, lateral root growth, rather than main root elongation, was presumably restricted (Figure 3, A and B). In cle2, cle3, and cle2cle3 mutants, the shoot/root mass ratio was comparable to that of WT plants.

**Root CLE genes are required for SUC2 expression in leaves**

To explore the effect of the CLE genes on root sucrose content at the molecular level, quantitative expression analysis

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**Figure 2** Carbohydrate levels in pCoYMV:CLE2 lines. The sucrose and glucose contents in roots and sucrose, glucose, and starch contents in mature leaves in pCoYMV:CLE2 lines are shown. The plants were grown on sucrose-free media. The center lines show the medians, and the box limits indicate the first to third quartiles. The whiskers indicate the maximum and minimum values, except outliers, and the crosses represent sample means. The dots represent individual measurements. Statistical differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett’s tests (*P < 0.05; **P < 0.01; ns, not significant compared to WT). Each result was obtained from 15 independent experiments.
of genes involved in sucrose transport, synthesis, and degradation in leaves and roots was conducted (sucrose efflux transport; SWEET11 and SWEET12, sucrose/H⁺ symport; SUC1 and SUC2, starch degradation; alpha-amylase-like 3 and beta-amylase 1, sucrose synthesis; fructose-1,6-bisphosphatase, and sucrose-phosphate synthase A1, sucrose degradation; cytosolic invertase 1, alkaline/neutral invertase C, and sucrose synthase 1). In the leaves and roots of the
prCoYMV:CLE2 and prCoYMV:CLE3 lines, no significant differences in the mRNA levels of these sucrose-related factors were observed (Supplemental Figure S9, P < 0.05). On the other hand, in cle1-7 leaves, the SUC2 mRNA level was reduced, while in cle2, cle3, and cle2cle3 mutants, the reduction was not observed (Figure 4A). SUC2 is known to play a key role in sucrose phloem loading (Gottwald et al., 2000), and mutations in SUC2 or the SWEET sucrose transporters result in a sucrose-dependent root growth-deficient phenotype (Gottwald et al., 2000; Chen et al., 2012). In addition, the SUC2 mRNA level in cle1-7 leaves was reduced, while in cle1, cle2, and cle2cle3 mutants, the reduction was not observed (Supplemental Figure S10). Alternatively, it is also possible that the promotion of sucrose degradation in roots can cause low sucrose levels in roots. To test this possibility, we performed grafting experiments to combine the roots and shoots of WT and cle1-7 plants (Figure 4C). We confirmed that grafted plants consisting of scions and rootstocks of the same genotype (WT/WT and cle1-7/cle1-7) showed SUC2 expression levels comparable to those of the corresponding intact plants. Then, we grafted cle1-7 scions onto WT rootstocks (cle1-7/WT) and WT scions onto cle1-7 rootstocks (WT/cle1-7). cle1-7/WT grafted plants showed SUC2 expression level intermediates of WT and cle1-7. Intriguingly, in WT/cle1-7 grafted plants, the SUC2 expression level in leaves was significantly lower than that in WT/WT grafted plants and comparable to that observed in cle1-7/cle1-7 grafted plants. This result indicates that the root genotype of the CLE genes is sufficient to regulate the expression level of SUC2 in leaves.

Although CLE1–7 were mainly expressed in roots (Figure 1D; Supplemental Figure S3), SUC2 expression in leaves was significantly decreased in cle1-7 (Figure 4A). Furthermore, the results from the mass spectrometric analysis and the peptide treatment experiments (Figure 1, A–C; Supplemental Figure S7) suggest that root-derived CLE peptides indeed affect shoots. To explore this possibility, we performed grafting experiments to combine the roots and shoots of WT and cle1-7 plants (Figure 4C). We confirmed that grafted plants consisting of scions and rootstocks of the same genotype (WT/WT and cle1-7/cle1-7) showed SUC2 expression levels comparable to those of the corresponding intact plants. Then, we grafted cle1-7 scions onto WT rootstocks (cle1-7/WT) and WT scions onto cle1-7 rootstocks (WT/cle1-7). cle1-7/WT grafted plants showed SUC2 expression level intermediates of WT and cle1-7. Intriguingly, in WT/cle1-7 grafted plants, the SUC2 expression level in leaves was significantly lower than that in WT/WT grafted plants and comparable to that observed in cle1-7/cle1-7 grafted plants. This result indicates that the root genotype of the CLE genes is sufficient to regulate the expression level of SUC2 in leaves.

**Figure 4** Relative mRNA levels of the genes related to sucrose transport, synthesis, and degradation. Relative mRNA levels in (A) leaves and (B) roots of cle mutants. C, Relative mRNA level of SUC2 in mature leaves of grafted plants. The center lines show the medians, and the box limits indicate the first to third quartiles. The whiskers indicate the maximum and minimum values, except outliers, and the crosses represent sample means. The dots represent individual measurements. Statistical differences were evaluated by one-way ANOVA followed by (A and B) Dunnett’s test (P < 0.05 compared to WT) or (C) Tukey’s test (P < 0.05). A and B, Each result is the mean ± SEM of measurements obtained from five independent experiments. C, The results were obtained from six independent experiments.
Discussion

In mammals, glucose is the primary energy source, and thus, the maintenance of appropriate glucose levels is critical for their survival. Mammalian peptide hormones, such as insulin and glucagon, play prominent roles in glucose homeostasis (Cryer, 2008). On the other hand, in plants, the roots are not only a major sink of carbon but also a source of water and inorganic nutrients. Therefore, ensuring an appropriate supply of photoassimilates to the roots and supporting their growth and development is essential for plant life to adapt to various conditions. To date, many studies have addressed the sucrose metabolism pathways in source and sink organs (Stitt and Zeeman, 2012; Ruan, 2014; Rossi et al., 2015) and the process of phloem loading of sucrose (Gottwald et al., 2000; Chen et al., 2012). However, the mechanism by which plants maintain carbon levels in roots has remained elusive.

Based on our findings, we propose a model in which CLE peptides function as a root-derived signal that systemically regulates sucrose content in the roots (Figure 5). In this model, the CLE peptides are translocated from roots to leaves via xylem and recognized by unknown receptor(s). The SUC2 sucrose transporter is a possible downstream factor and may contribute to enhanced sucrose translocation to the roots. The CLE peptides thus fine-tune the sucrose level in roots and maintain their growth. In the field, light conditions are likely to fluctuate diurnally, and consequently, plants must distribute their unstable supply of sucrose effectively. This mechanism will likely be critical, particularly for plants facing such fluctuating energy stress.

In soybean, XAP4/GmCLE32 strongly responded to low-carbon conditions (Supplemental Figure S1). Our previous RNA sequence analysis suggests that the mRNA level of XAP4/GmCLE32 is low under normal conditions (Okamoto et al., 2015). On the other hand, based on the Arabidopsis eFP browser (http://bar.utoronto.ca/efp2/Arabidopsis/Arabidopsis_eFPBrowser2.html), the basal mRNA level of CLE2 in roots seems to be comparably high. Therefore, once XAP4/GmCLE32 responds to low-carbon conditions, its upregulation rate may become higher than that of Arabidopsis CLE2. The amino acid sequence of the XAP4/GmCLE32 peptide is almost identical to that of Arabidopsis CLE1–7 (Supplemental Figure S2). In addition, the XAP4/GmCLE32 peptide was identified from soybean xylem exudate (Okamoto et al., 2015). Although its gain-of-function and loss-of-function phenotypes remain to be clarified, these findings imply that a similar molecular mechanism exists in other apoplastic-loading plants.

However, it is important to note that CLE peptides are reported to function in the regulation of lateral root development (Araya et al., 2014). The growth and development of organs require additional energy and resources for the de novo synthesis of cellular components. This finding raises the possibility that excessive formation of lateral roots can lead to a decrease in the sucrose contents in cle1-7 mutants (Figure 5). Although the transient application (24 h) of CLE2 peptide to the leaves of WT or cle1-7 mutant plants enhanced their root sucrose content (Supplemental Figures S7 and S8) and may not affect root morphology, the possibility cannot be eliminated. Furthermore, this possibility appears to be compatible with the former hypothesis; under carbon-deficient conditions, CLE peptides might function to conserve energy by restricting lateral root growth in parallel with demanding sucrose to leaves. To understand the mechanism through which the CLE peptide affects sucrose levels, its receptor(s) in this pathway should be identified.

In this study, we focused on root sucrose status to evaluate the effect of CLEs. Sucrose is the major photoassimilate that is transported from source leaves, and in roots, sucrose is degraded and used as an energy or carbon source for various biological processes. In our analyses, although variation in the root glucose levels was detected, these levels tended to be higher in prCoYMV/CLE2 and prCoYMV/CLE3 lines and CLE2 peptide-applied plants than in control plants (Figure 2; Supplemental Figures S4B and S7). Considering that root glucose is mainly derived from sucrose degradation and is also metabolized into other carbon compounds, CLEs might affect root carbon metabolism, including the secretion of carbon sources into the rhizosphere.

Our loss-of-function analysis raises the possibility that functional redundancy among CLE1–7 exists in the positive effect on of sucrose content in roots (Supplemental Figure S4A). RT-qPCR analysis showed that the mRNA levels of CLE2 and CLE3 were upregulated under low-carbon conditions (Supplemental Figure S4A). However, the mechanism by which these CLE peptides act on the root sucrose metabolism requires further investigation. Additionally, the role of CLE peptides in the regulation of lateral root growth and the possible involvement of other CLE peptides in this process should be explored in future studies.
conditions (Figure 1D; Supplemental Figure S3). This suggests that one role of the CLE2 and CLE3 peptides is a carbon-deficient signal to demand sucrose from shoots. Considering that the amino acid sequences of (putative) mature CLE1–7 peptides are almost identical, CLE1–7 peptides would have the same biological activity. Based on the Arabidopsis RNA-seq database (http://ipfs.sustech.edu.cn/pub/athrna/), the fragments per kilobase of transcript per million mapped reads values of the CLE1 and CLE6–7 genes appear to be comparable to those of the CLE2 and CLE3 genes. Therefore, it appears that the basal mRNA level of those CLE genes masks the lack of CLE2 and/or CLE3 genes and that the phenotypes were not observed in cle2, cle3, or cle2cle3 mutants.

CLE1, CLE3, CLE4, and CLE7 have been shown to respond to nitrogen-poor conditions (Araya et al., 2014). It is known that nitrogen deficiency causes sucrose accumulation in roots (Scheible et al., 1997); thus, these CLE peptides might facilitate sucrose allocation to roots under nitrogen-deficient conditions. In contrast, it has also been reported that the mRNA levels of all CLE genes except CLE3 are upregulated by the transient application of nitrate (Ma et al., 2020). Although additional research is needed to further clarify the responses of CLE1–7 genes to nitrogen, these CLE genes might mediate some relationships between nitrogen and carbon status in roots. To date, it is known that various environmental factors affect carbon allocation in plants (Hermans et al., 2006; Lemoine et al., 2013), and it has been reported that the mRNA levels of CLE2, CLE3, CLE4, and CLE7 are affected by some environmental stimuli, such as salt, cold, and low sulfate conditions (Dong et al., 2019; Ma et al., 2020). Furthermore, the mRNA level of CLE2 is affected by TOR, which integrates nutrients, energy, and environmental inputs to regulate cell proliferation and growth (Menand et al., 2002; Baena-González et al., 2007; Robaglia et al., 2012; Xiong et al., 2013). Taken together, CLE2 and its homologs may control root sucrose levels in response to various environmental stimuli.

Low sucrose content in leaves was observed in both cle1-7 and cle2cle3 (Supplemental Figure S4A). However, a decrease in root sucrose content was observed only in cle1-7 and not in cle2cle3. This suggests that the CLE genes affect the sucrose contents of roots and leaves independently. In cle1-7, the mRNA level of SUC2 in leaves was decreased. Mutations in sucrose transporters impair the phloem loading of sucrose and lead to a sucrose-dependent root growth-deficient phenotype (Gottwald et al., 2000; Chen et al., 2012), and there is a partial association between SUC2 mRNA levels and root growth (Srivastava et al., 2009). Considering these reports, modest downregulation of SUC2 mRNA levels in cle1-7 may be linked to its phenotypes in root sucrose level and root growth (Supplemental Figure S4A; Figures 3 and 4, A). However, in the prCoYMV:CLE2 and prCoYMV:CLE3 lines, the SUC2 mRNA level was not affected (Supplemental Figure S9). Xu et al. (2018) reported that the upregulation of sucrose transporters is likely caused by posttranscriptional regulation. It has been reported that energy-dependent sucrose symporters are positively regulated by their ubiquitination and phosphorylation, protein interaction, and H+ gradient between the extracellular and intracellular space (Boorer et al., 1996; Fan et al., 2009; Xu et al., 2020). To determine whether SUC2 is a downstream target of CLEs, the effect of CLEs on SUC2 posttranscriptional regulation and the genetic relationship between CLE genes and SUC2 should be examined.

Mutations in SUC2 or SWEET transporters lead not only to a decrease in sucrose contents in the phloem but also to an increase in starch accumulation in the leaves (Gottwald et al., 2000; Chen et al., 2012). This would be caused by defects in the ability of sucrose translocation from source leaves to sink organs. In contrast, in cle1-7, the starch contents in leaves were not affected (Supplemental Figure S4A). Considering that CLE peptides function as signaling molecules, it is possible that they have multiple sites of action. Although our reverse transcription quantitative PCR (RT-qPCR) analysis showed that the mRNA level of some sucrose-related genes was not affected by loss-of-function CLE genes (Figure 4; Supplemental Figure S9), we cannot eliminate the possibility that not only SUC2 but also multiple factors that are related to carbohydrate metabolism or photosynthesis might be affected by CLEs. Ma et al. (2020) discussed the possibility that CLE2 may regulate carbon metabolism. Further analysis is needed to understand the effects of CLEs on these pathways.

Materials and methods

Plant materials and growth conditions

Arabidopsis (A. thaliana) ecotype Columbia-0 and CRISPR/Cas9-induced cle2 (cle2-cr1), cle3 (cle3-cr1), cle2cle3 double, and cle1-7 septuple mutants (Yamaguchi et al., 2017), prCoYMV:CLE2 and prCoYMV:CLE3 lines were used in this study. The cle2cle3 double mutant was generated by crossing cle2-cr1 and cle3-cr1. The cle1-7 septuple mutant was isolated by mutating cle7-cr1, with the mutation position shown in Supplemental Figure S4.

Seeds were sterilized and sown on 1/2 MS media (1/2 MS, 0.8% (w/v) agar, 1% (w/v) sucrose) and then incubated in controlled conditions (23°C, 10-h light/14-h dark, photosynthetic photon flux density (PPFD) = 100 μmol m−2s−1) for 7 days. For RT-qPCR analysis and sugar and starch quantification, plants at 7 DAG were transferred to sucrose-free 1/2 MS media (1/2 MS, 0.8% agar) and grown for 10 days under controlled conditions (23°C, 10-h light/14-h dark, PPFD = 280 μmol m−2s−1). The plants were sampled at the end of the day. The samples were immediately wiped with soft paper several times to remove water that adhered to the samples and stored at −80°C. For the growth assay, plants at 7 DAG were transferred to either 1% sucrose 1/2 MS media or sucrose-free media (1/2 MS, 0.8% agar). The plants were grown for 10 days under normal light (constant light intensity) conditions (23°C, 10 h [PPFD = 280 μmol m−2s−1]/dark 14 h) or grown for 5 days under normal light.
conditions and thereafter for 5 days under diurnal fluctuating light conditions (23°C, light 1.5 h (PPFD = 20 μmol m⁻² s⁻¹), 2 h (PPFD = 150 μmol m⁻² s⁻¹), 3 h (PPFD = 280 μmol m⁻² s⁻¹), 2 h (PPFD = 150 μmol m⁻² s⁻¹), 1.5 h (PPFD = 20 μmol m⁻² s⁻¹)/dark 14 h).

*Glycine max* cv. Enrei was used in this study. Soybean seeds were surface sterilized with 70% ethanol for 1 min and rinsed with distilled water. The seeds were sown on vermiculite and grown under constant light conditions (23°C, 16-h light/8-h dark, PPFD = 400 μmol m⁻² s⁻¹). Shoot excision, decapitation, and defoliation were conducted using plants at 18 DAG. For sucrose or mannose application, the remaining petiole was inserted into a 1.5-mL tube whose tip was cut and filled with 300-mM sucrose or mannose. Eight hours after the treatments, the roots were sampled.

**Analysis of Arabidopsis xylem sap**
Sterilized seeds were sown on MS media and grown in the dark for 4 days. Then, the plants were transferred to 10-h/14-h light/dark cycles (PPFD = 100 μmol m⁻² s⁻¹) for 5 days. The plants were then transferred to sucrose-free media and grown for 14 days. The xylem exudate was collected by cutting the hypocotyl with a razor at the end of the night. The exudate was collected for 4 h, and ~320 μL of exudate was obtained from 360 plants. The xylem exudate was partially purified via o-chlorophenol extraction and acetone precipitation as described in *Ohyama et al.* (2009), and precipitated materials were resuspended in 50 μL of water.

Nano-LC–MS analysis was performed using an EASY-nLC 1000 (Thermo Fisher Scientific, Waltham, MA, USA) connected to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Aliquots of small peptide fractions (5 μL) were loaded onto a trapping column (75 μm i.d. × 20 mm, Acclaim PepMap 100 C18 LC; Thermo Fisher Scientific). Peptides were subsequently eluted from the trapping column and separated on a nanocapillary column (75 μm i.d. × 125 mm, C18; Nikkko Technos, Tokyo, Japan), with a 10-min 0%–30% acetonitrile (containing 0.1% formic acid) gradient, followed by a 2-min 30%–80% gradient, with a final 8-min isocratic step at 80% acetonitrile at a flow rate of 300 nL min⁻¹. The nano-HPLC eluate was introduced into a mass spectrometer via an electrospray ionization interface at a spray voltage of 2.0 kV. The mass spectrometer was operated in positive ion mode with a capillary temperature of 330°C. Mass spectra were obtained by scanning from m/z 150 to m/z 2,000. Nano-LC–MS/MS analysis was performed by selecting the indicated molecular ion as the precursor ion at 30% normalized collision energy using the higher energy collision dissociation mode.

**Photosynthesis inhibiting treatments**
For DCMU treatment, plants at 17 DAG were sprayed with 50 μM DCMU in 0.1% ethanol or mock solution (0.1% ethanol) at the end of the night and further incubated for 6 h under 280 μmol m⁻² s⁻¹ light. For dark treatment, plants at 16 DAG were incubated in the dark for 24 h.

**RT-qPCR analysis**
Total RNA was extracted from the shoots and roots of 17-DAG plants using an RNeasy Plant kit (Qiagen, Hilden, Germany), and cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) following the manufacturer’s protocol. RT-qPCR analysis was performed using Thunderbird SYBR qPCR Mix (Toyobo), and PCR cycling conditions were as follows: a denaturation step at 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. RT-qPCR was performed using the CFX Connect Real Time PCR detection system (BIO-RAD, Hercules, CA, USA). ACTIN2 (AT3G18780) was used as a reference gene. The primers used for RT-qPCR are listed in *Supplemental Table S1*. 

**Transformation of plants**
For gain-of-function analyses of CLE2 and CLE3, the CoYMV promoter was used (*Medberry et al.*, 1992). The CoYMV promoter region was amplified by PCR with the primers listed in *Supplemental Table S1*. PCR was performed using Prime Star GXL (Takara, Shiga, Japan) according to the manufacturer’s protocol. The reaction program was as follows: initial denaturation (98°C for 2 min), 35 cycles of amplification (98°C for 10 s, 60°C for 15 s, and 68°C for 1.5 min) and one extension step (68°C for 5 min), followed by cooling of the reaction to 4°C. The PCR product was introduced into the HindIII and SacI sites of pBI101 using the In-Fusion system (Clontech, Mountain View, CA, USA). This vector was named pBI-CoYMV. The coding sequences of CLE2, CLE3, and GUS, which were derived from pBI121, were amplified by PCR with the primers listed in the *Supplemental Table S1*. PCR was performed as mentioned above. The PCR products were cloned into the BamHI and Sall sites of pBI-CoYMV using the In-Fusion system (Clontech).

*Arabidopsis thaliana* Columbia-0 was transformed using *Agrobacterium tumefaciens* AGL1, which harbored prCoYMV:CLE2, prCoYMV:CLE3 or prCoYMV:GUS, via the floral dip method (*Clough and Bent*, 1998). Transformants were selected using 50 μg mL⁻¹ kanamycin.

**Quantification of the nonstructural carbohydrate content**
Quantification of glucose, sucrose and starch contents in mature leaves (L1–4) and roots was conducted based on *Araya et al.* (2006). Frozen samples were ground with a Freeze Crusher μT-48 (TAITEC) using metal cones at 1,250 r.p.m. for 30 s. After adding 1 mL of 80% ethanol, the suspension was incubated 3 times at 80°C for 10 min, and the precipitate was used for the determination of starch. The supernatant was concentrated with a centrifuge. Up to 150 μL of distilled water and chloroform were added to the concentrated supernatant to separate sugars from soluble proteins. After centrifugation, the upper aqueous phase was used for the determination of glucose and sucrose levels. Thirty microliters of the solution was incubated with 50 μL of invertase (Wako, Richmond, VA, USA) to break down sucrose into glucose and fructose. The starch in the precipitate
was suspended in distilled water and boiled for 60 min. An equal volume of amyloglucosidase was added to the boiled suspension and incubated for 60 min at 55°C. The mixture was centrifuged, and the upper aqueous phase was used for the determination of starch. A glucose-C2-test (Wako) was used for detection.

Peptide treatment
One mature rosette leaf from 16 DAG plants was pinched once with forceps, and a 2 μL drop of 10-nM peptide solution or H2O was placed on the wound site at the end of the day. After 24 h, the treated leaves were removed, and the remaining shoots (without the treated leaves) and roots were frozen in liquid nitrogen to be analyzed separately.

Grafting experiment
Sterilized seeds were sown on MS media and grown under low light conditions (PPFD = 50 μmol m−2s−1, 23°C) for 4 days. Arabidopsis seedlings were cut at the hypocotyl using a surgical needle. Then, scions and rootstocks were inserted into sterilized short silicon tubes (length ~2 mm, φ0.4 mm) to make contact with each other. The grafted seedlings were grown on sucrose-free media under low light conditions (PPFD = 25 μmol m−2s−1, 27°C) for 4 days. Then, the plate was transferred to PPFD = 50 μmol m−2s−1, 23°C. After 2 days, grafted plants were transferred to new sucrose-free media and grown for 7 days in PPFD = 100 μmol m−2s−1, 23°C. The plants were grown for an additional 7 days under PPFD = 280 μmol m−2s−1, 23°C. In all steps, the plants were grown under short-day conditions (10-h light/14-h dark).

Accession numbers
The accession numbers of the genes analyzed in this study are listed in Supplemental Table S1.

Supplemental data
The following materials are available in the online version of this article.
Supplemental Figure S1. XAP4/GmCLE32 expression in soybean roots.
Supplemental Figure S2. Homologs of XAP4/GmCLE32.
Supplemental Figure S3. CLE1 and CLE3–7 expression under low-carbon conditions.
Supplemental Figure S4. Carbohydrate levels in cle knockout mutants and pCoYMV/CLE3 lines.
Supplemental Figure S5. Mutations of the cle1-7 septic mutant.
Supplemental Figure S6. Relative mRNA level of CLE2 or CLE3 in the transgenic lines.
Supplemental Figure S7. Carbohydrate levels in WT plants treated with CLE2 peptide.
Supplemental Figure S8. Carbohydrate levels in cle1-7 mutant plants treated with CLE2 peptide.

Supplemental Figure S9. Relative mRNA levels of the genes related to sucrose transport, synthesis, and degradation.
Supplemental Figure S10. Relative mRNA level of SUC2.
Supplemental Table S1. Primers list.

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References
Araya T, Miyamoto M, Wibowo J, Suzuki A, Kojima S, Tsuchiya YN, Sawa S, Fukuda H, von Wirén N, Takahashi H (2014) CLE-CLAVATA1 peptide-receptor signaling module regulates the expansion of plant root systems in a nitrogen-dependent manner. Proc Natl Acad Sci USA 111: 2029–2034
Araya T, Noguchi K, Terashima I (2006) Effects of carbohydrate accumulation on photosynthesis differ between sink and source leaves of Phaseolus vulgaris L. Plant Cell Physiol 47: 644–652
Baena-González E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signalling. Nature 448: 938–942
Bellafiore S, Barneche F, Peltier G, Rochaix J-D (2005) State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. Nature 433: 892–895
Boorer KJ, Loo DD, Frommer WB, Wright EM (1996) Transport mechanism of the cloned potato H+/sucrose cotransporter StSUT1. J Biol Chem 271: 25139–25144
Chen LQ, Xu QX, Hou BH, Sosso D, Osorio S, Fernie AR, Frommer WB (2012) Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. Science 335: 207–211
Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743
Cryer P (2008) Glucose homeostasis and hypoglycemia. Williams Textbook of Endocrinology, 11th edn. Saunders, Philadelphia, PA, pp 1503–1533
Cle-CLAVATA1 signaling pathway modulates lateral root development under sulfur deficiency. Plants 8: 103

Fan RC, Peng CC, Xu YH, Wang XF, Li Y, Shang Y, Du SY, Zhao R, Zhang XY, Zhang LY, et al. (2009) Apple sucrose transporter SUT1 and sorbitol transporter SOT6 interact with cytochrome b5 to regulate their affinity for substrate sugars. Plant Physiol 150: 1880–1901

Fletcher JC (2020) Recent advances in Arabidopsis CLE peptide signaling. Trend Plant Sci 25: 1005–1016

Giaquinta RT (1983) Phloem loading of sucrose. Ann Rev Plant Physiol 34: 347–387

Gottwald JR, Krysan PJ, Young JC, Evert RF, Sussman MR (2000) Genetic evidence for the in planta role of phloem-specific plasma membrane sucrose transporters. Proc Natl Acad Sci USA 97: 13979–13984

Hastwell AH, Gresshoff PM, Ferguson BJ (2015) Genome-wide annotation and characterization of CLAVATA/ESR (CLE) peptide hormones of soybean (Glycine max) and common bean (Phaseolus vulgaris), and their orthologues of Arabidopsis thaliana. J Exp Bot 66: 5271–5287

Hermans C, Hammond JP, White PJ, Verbruggen N (2006) How do plants respond to nutrient shortage by biomass allocation? Trend Plant Sci 11: 610–617

Ito Y, Nakamoto I, Motose H, Iwamoto K, Sawa S, Dohmae N, Fukuda H (2006) Dodeca-CLE peptides as suppressors of plant stem cell differentiation. Science 313: 842–845

Jones DL, Nguyen C, Finlay RD (2009) Carbon flow in the rhizosphere: carbon trading at the soil–root interface. Plant Soil 321: 5–33

Jun J, Fiume E, Roeder AHK, Meng L, Sharma VK, Osmont KS, Baker C, Ha CM, Meyerowitz EM, Feldman LJ, et al. (2010) Comprehensive analysis of CLE polypeptide signaling gene expression and overexpression activity in Arabidopsis. Plant Physiol 154: 1721–1736

Kondo T, Sawa S, Kinoshita A, Mizuno S, Kakimoto T, Fukuda H, Sakagami Y (2006) A plant peptide encoded by CLV3 identified by in situ MALDI-TOF MS analysis. Science 313: 845–848

Lemoine R, La Camera S, Atanassova R, Dédaile-champ F, Allario T, Portaut N, Bonnemain JL, Laloi M, Coutous-Thévenot P, Maurousset L, et al. (2013) Source-to-sink transport of sugar and regulation by environmental factors. Front Plant Sci 4: 272

Lincoln T, Eduardo Z, editors (2018) Transport and Translocation of Water and Solutes. Plant Physiology and Development, 6th edn. Oxford University Press, Oxford, pp 81–168

Ma D, Endo S, Betsuyaku S, Shimotoho A, Fukuda H (2020) CLE2 regulates light-dependent carbohydrate metabolism in Arabidopsis shoots. Plant Mol Biol 104: 561–574

McCormick AJ, Kruger NJ (2015) Lack of fructose 2,6-bisphosphate compromises photosynthesis and growth in Arabidopsis in fluctuating environments. Plant J 81: 670–683

Meddery SL, Lockhart BE, Olszewski NE (1992) The Commelina yellow mottle virus promoter is a strong promoter in vascular and reproductive tissues. Plant Cell 4: 185–192

Menand B, Desnos T, Nussaume L, Berger F, Bouchez D, Meyer C, Robaglia C (2002) Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene. Proc Natl Acad Sci USA 99: 6422

Ohyama K, Shinohara H, Ogawa-Ohashi M, Matsubayashi Y (2009) A glycopeptide regulating stem cell fate in Arabidopsis thaliana. Nat Chem Biol 5: 578–580

Okamoto S, Shinohara H, Mori T, Matsubayashi Y, Kawaguchi M (2013) Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase. Nat Commun 4: 2191

Okamoto S, Suzuki T, Kawaguchi M, Higashiyama T, Matsubayashi Y (2015) A comprehensive strategy for identifying long-distance mobile peptides in xylem sap. Plant J 84: 611–620

Robaglia C, Thomas M, Meyer C (2012) Sensing nutrient and energy status by SnRK1 and TOR kinases. Curr Opin Plant Biol 15: 301–307

Rossi M, Bermudez L, Carrari F (2015) Crop yield: challenges from a metabolic perspective. Curr Opin Plant Biol 25: 79–89

Ruan YL (2014) Sucrose metabolism: gateway to diverse carbon use and sugar signaling. Annu Rev Plant Biol 65: 33–67

Scheible WR, Lauerer M, Schulze ED, Caboche M, Stitt M (1997) Accumulation of nitrate in the shoot acts as a signal to regulate shoot-root allocation in tobacco. Plant J 11: 671–691

Srivastava AC, Ganesan S, Ismail IO, Ayre BG (2009) Effective carbon partitioning driven by exotic phloem-specific regulatory elements fused to the Arabidopsis thaliana AtSUC2 sucrose-proton symporter gene. BMC Plant Biol 9: 7

Stadler R, Sauer N (1996) The Arabidopsis thaliana AtSUC2 gene is specifically expressed in companion cells. Bot Acta 109: 299–306

Stitt M, Zeeman SC (2012) Starch turnover: pathways, regulation and role in growth. Curr Opin Plant Biol 15: 282–292

Takahashi F, Suzuki T, Osakabe Y, Betsuyaku S, Kondo Y, Dohmae N, Fukuda H, Yamaguchi-Shinozaki K, Shinozaki K (2018) A small peptide modulates stomatal control via abscisic acid in long-distance signalling. Nature 556: 235–238

Truenrit E, Sauer N (1995) The promoter of the Arabidopsis thaliana SUC2 sucrose-H+ symporter gene directly expresses beta-glucuronidase to the phloem: evidence for phloem loading and unloading by SUC2. Planta 196: 564–570

Vaughn MW, Harrington GN, Bush DR (2002) Sucrose-mediated transcriptional regulation of sucrose symporter activity in the phloem. Proc Natl Acad Sci USA 99: 10876–10880

Xiong Y, McCormack M, Li L, Hall Q, Xiang C, Sheen J (2013) Glucose–TOR signalling reprograms the transcriptome and activates meristems. Nature 496: 181–186

Xu Q, Chen S, Yunjuan R, Chen S, Liesche J (2018) Regulation of sucrose transporters and phloem loading in response to environmental cues. Plant Physiol 176: 930–945

Xu Q, Yin S, Ma Y, Song M, Song Y, Mu S, Li Y, Liu X, Ren Y, Gao C, et al. (2020) Carbon export from leaves is controlled via ubiquitination and phosphorylation of sucrose transporter SUC2. Proc Natl Acad Sci USA 117: 6223–6230

Yamaguchi YL, Ishida T, Sawa S, Yamaguchi YL, Ishida T, Yoshimura M, Imamura Y, Shimaoka C, Xu Q, Chen S, Yunjuan R, Chen S, Liesche J (2013) Crop yield: challenges from a metabolic perspective. Curr Opin Plant Biol 16: 1848–1867

Yamaguchi YL, Ishida T, Yoshimura M, Imamura Y, Shimaoka C, Sawa S (2017) A collection of mutants for CLE-peptide-encoding genes in Arabidopsis generated by CRISPR/Cas9-mediated gene targeting. Plant Cell Physiol 58: 1848–1856

Zhang Z, Liu L, Kucukoglu M, Tian D, Larkin RM, Shi X, Zheng B (2020) Predicting and clustering plant CLE genes with a new method developed specifically for short amino acid sequences. BMC Genomics 21: 709