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*Project Report*

# *CLAVATA3/EMBRYO SURROUNDING REGION* Genes Involved in Symbiotic Nodulation in *Pisum sativum*

**Maria A. Lebedeva [1,2,*], Darina S. Sadikova [1], Daria A. Dobychkina [1,2], Vladimir A. Zhukov [1,3] and Lyudmila A. Lutova [1,2]**

[1] Department of Genetics and Biotechnology, Saint Petersburg State University, Saint Petersburg 199034, Russia
[2] Center for Genetic Technologies, N. I. Vavilov All-Russian Institute of Plant Genetic Resources (VIR), Saint Petersburg 190000, Russia
[3] Laboratory of Genetics of Plant-Microbe Interactions, All-Russia Research Institute for Agricultural Microbiology (ARRIAM), Saint Petersburg 196608, Russia
* Correspondence: m.a.lebedeva@spbu.ru

**Abstract:** CLE (CLAVATA3/Embryo Surrounding Region) peptides regulate different aspects of plant development. In legumes, CLE peptides are known as key components of autoregulation of nodulation (AON), which systemically controls the number of nitrogen-fixing nodules formed on the root upon symbiotic interaction with soil bacteria rhizobia. CLE peptides are produced in the root in response to rhizobia inoculation and are transported via xylem to the shoot, where they are recognized by a specific receptor. As a result, a subsequent nodule development is suppressed by a negative feedback mechanism. In addition, nitrate-induced *CLE* genes have been identified in model legumes, which mediate nitrate-dependent inhibition of nodulation. However, little is known about the functions of nodulation-related CLE peptides, which have not been studied in *Pisum sativum*. Here, we studied four homologues of *CLE* genes in *Pisum sativum*, which are closely related to nodulation-suppressing *CLEs* from other legumes. The expression levels of these genes were increased in developing nodules. Among them, *PsCLE13*, *PsCLE12*, and the *PsNIC*-like genes were upregulated in response to nitrate treatment. Moreover, we found that overexpression of the *PsCLE13* and *PsCLE12* genes resulted in the decreased nodule number on transgenic roots. The expression levels of pea homologues of the *TOO MUCH LOVE (TML)* genes were upregulated in *PsCLE13*- and *PsCLE12*-overexpressing roots in comparison with the control (GUS-overexpressing) roots, suggesting that inhibitory effect of PsCLE13 and PsCLE12 is mediated through the induction of the *PsTML* genes.

**Keywords:** CLE; nitrate; legume–rhizobia symbiosis; AON (autoregulation of nodulation)



**Citation:** Lebedeva, M.A.; Sadikova, D.S.; Dobychkina, D.A.; Zhukov, V.A.; Lutova, L.A. *CLAVATA3/EMBRYO SURROUNDING REGION* Genes Involved in Symbiotic Nodulation in *Pisum sativum*. *Agronomy* **2022**, *12*, 2840. https://doi.org/10.3390/agronomy12112840

Academic Editor: C. Eduardo Vallejos

Received: 23 August 2022
Accepted: 4 November 2022
Published: 14 November 2022



## 1. Introduction

Legume plants interact symbiotically with soil bacteria rhizobia to initiate the formation of nitrogen-fixing nodules. The development of symbiotic nodules is governed by a systemic control, i.e., at the whole-plant level, to balance with the host plant resources and nutrient supply. Such systemic regulation is implemented via AON (autoregulation of nodulation) and nitrate-dependent inhibition of nodulation [1]. In developing nodules, the NIN (NODULE INCEPTION) transcription factor, a master regulator of symbiotic nodulation, triggers the expression of the *CLE* (*CLAVATA3/EMBRYO SURROUNDING REGION*) genes [2,3]. The mature products of these genes are the CLE peptides, consisting of 12–13 amino acids and derived from the CLE domains of CLE precursor proteins. These peptides are transported from the root to the shoot, where they are recognized by a shoot-acting receptor complex [4]. The shoot-acting receptor of the CLE peptides is the LRR-RLK kinase (SUNN (SUPERNUMERIC NODULE) in *Medicago truncatula Gaertn.*, HAR1 (HYPERNODULATION ABERRANT ROOT FORMATION) in *Lotus japonicas*, and SYM29 in pea

(*Pisum sativum* L.)) [5–9], which has high sequence similarity with the CLV1 kinase operating in the shoot apical meristem [5]. Being activated by the root-derived CLE peptides, the LRR-RLK kinase triggers a feedback response that inhibits nodulation on the root. Several nodulation-related CLE peptides have been described in model legumes. They include LjCLE-RS1 (CLE-ROOT SIGNAL 1), LjCLE-RS2 and LjCLE-RS3 in *Lotus japonicus* (Regel.) K. Larsen [6,7], MtCLE13, MtCLE12, MtCLE35 in *M. truncatula* and GmRIC1 (RHIZOBIUM INDUCED CLE 1), GmRIC2, GmNIC1 (NITRATE INDUCED CLE 1), and GmNIC2) in soybean (*Glycine max* (L.) Merr.). The overexpression of corresponding nodulation-related *CLE* genes in legumes inhibited symbiotic nodulation and significantly reduced nodule number [4,10–14].

Activation of the shoot-acting CLV1-like receptor kinase by root-derived CLE peptides triggers feedback signaling cascade inhibiting nodulation. Among downstream targets of this pathway, shoot-to-root transported microRNA *miR2111* was identified: the amount of this microRNA was decreased in response to CLE-LRR-RLK activation [15,16]. microRNA *miR2111* downregulates the transcript levels of the *TML* (*TOO MUCH LOVE*) genes in the root, which encode F-box proteins, characterized as the negative regulator of nodulation [15,16]. Activation of the CLE-LRR-RLK pathway downregulates miR2111 synthesis in the shoot resulting in the upregulation of *TML* expression in the root, thereby inhibiting nodulation [15,17].

The development of nodules is an energy-consuming process. Therefore, legume plants form symbiotic nodules only when there is a lack of nitrogen source in soil, whereas high concentrations of nitrate inhibit nodulation. Nitrate-dependent inhibition of nodulation is also mediated by root-produced CLE peptides. The NLP (NIN-LIKE PROTEINS) transcription factors were shown to activate the expression of nitrate-inducible *CLE* genes as well as other nitrate-regulated genes in plants. In model legumes, a set of *CLE* genes was shown to be activated by both rhizobia and nitrate (for example, *LjCLE-RS2, LjCLE-RS3* [10,18], and *MtCLE35* [12–14], whereas other *CLE* genes (including *LjCLE-RS1* [10], *MtCLE13* and *MtCLE12* [11], and *GmRIC1* and *GmRIC2* [9]) are induced only upon nodulation and are not responsive to nitrate treatment.

The functions of nodulation-related *CLE* genes in *P. sativum* have not been studied to date. Previously, we identified 45 *CLE* gene family members in pea [19]. Here, we focused on four pea *CLE* genes that have the higher similarity to nodulation-related *CLEs* from other legumes. We examined the expression levels of these genes in developing nodules, studied the effect of nitrate on their expression, and described the inhibitory effect of overexpression of the *PsCLE13* and *PsCLE12* genes on nodulation. Our results suggest that these two genes play a role in AON and act as negative regulators of nodulation, possibly via modulation of the expression level of pea homologs of *TOO MUCH LOVE (TML)* genes.

## 2. Materials and Methods

### *2.1. Plant Material, Bacterial Strains, and Growth Conditions*

*P. sativum* cv. Frisson seeds were surface sterilized with concentrated sulfuric acid for 8 min, washed five times with water, transferred to 1% water agar plates, and germinated at room temperature in the dark for 3–4 days. For expression analysis, plants were grown in aeroponics system containing aeroponic nutrient medium [20]. Seven days after germination, plants were inoculated with *Rhizobium leguminosarum* bv. viciae RCAM1026 (ARRIAM, WDCM 966 [21]) culture grown on YMB (Yeast Extract Mannitol) plates (diluted to the final concentration $OD_{600}$ = 0.05). Non-inoculated control roots, together with the inoculated roots, were harvested and used for RNA extraction. For each time point, the roots from three different plants were combined in one sample for RNA extraction. The experiment was repeated three times.

### *2.2. Nitrate Treatment*

Nitrate treatment of pea plants was performed in a hydroponic system. First, plants were grown on plates containing Fahraeus medium for 5 days and then were transferred

to the hydroponic system, containing nitrogen-free HY medium [22]. After 3 days, $KNO_3$ was added to the medium to a final concentration of 10 mM. Plant roots were harvested 24 and 48 h after $KNO_3$ treatment and used for gene expression analysis, and untreated plants were used as a control. RNA was extracted from individual roots; 4–5 biological replicates were used for each treatment. Two additional independent biological experiments on nitrate treatment were performed, where 3–5 biological replicates were analyzed for each treatment group in each experiment.

### *2.3. Molecular Cloning*

The CDS sequences of *PsCLE12* and *PsCLE13* genes were amplified using high fidelity Phusion polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with attB sites added (underlined) to the forward and reverse primers (PsCLE12_FOR: AAAAAAGCAGGCTTCATGGAGAATACAAGTAATATA; PsCLE12_REV:CAAGAAAGCTGGGTTTCAAGATGGACCTGATTATT; PsCLE13_FOR: AAAAAAGCAGGCTTCATGGGTCGGTATACAAATCAAGTG; PsCLE13_REV: CAAGAAAGCTGGGTTTTACTTGCTTGGTGGTGATTTTC). The PCR products were cloned into the entry vector pDONR207 (Thermo Fisher Scientific, Waltham, MA, USA) via BP Clonase™ enzyme (Thermo Fisher Scientific, Waltham, MA, USA) and then into the destination vector pB7WG2D (containing 35S promoter for overexpression and GFP cassette to select transgenic plants under fluorescence) using LR Clonase™ II enzyme (Thermo Fisher Scientific, Waltham, MA, USA) The constructs were checked by sequencing and restriction analysis. The resulting vectors (p35S::PsCLE12 and p35S::PsCLE13) were introduced into *Agrobacterium rhizogenes* strain ArQUA.

### *2.4. Agrobacterium Rhizogene-Mediated Pea Transformation*

*Agrobacterium rhizogene*-mediated pea transformation was carried out by stabbing the hypocotyls as described in [23]. *A. rhizogenes* Arqua strain bearing the 35S:GUS construct was used as a control. As a result, composite plants were obtained with emerged transgenic roots at the infection site. Plants were transferred into pots with vermiculite, watered with nitrogen-free Farhaeus medium, grown for approximately 7 d at 21 °C with a 16-h photoperiod, and subsequently inoculated with *R. leguminosarum* bv. viciae RCAM1026. Rhizobia culture was grown on YMB (Yeast Extract Mannitol) plates and resuspended in liquid Farhaeus medium to the final concentration $OD_{600}$ = 0.5–0.7, and 1 mL of suspension was used for inoculation of each plant. Transgenic roots were selected based on GFP detection using fluorescence stereo microscope Leica M205 FA (www.leica-microsystems.com, accessed on 22 October 2022, Leica Microsystems, Wetzlar, Germany) or Zeiss SteREO Discovery.V12 (Carl Zeiss Microscopy GmbH, Germany). The images were processed using the ImageJ program (National Institutes of Health, Bethesda, MD, USA) [24]. The experiment was repeated three times, with 10–15 plants for each group analyzed in one biological experiment.

### *2.5. RNA Extraction and cDNA Synthesis*

Total RNA was extracted from plant roots using TRIZol reagent according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). DNase treatment was performed using Rapid Out DNA Removal Kit (Thermo Fisher Scientific, Waltham, MA, USA). NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to measure RNA concentration and quality.

### *2.6. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis*

qRT-PCR analysis was performed using the C1000 thermal cycler with CFX-96 real-time PCR detection system (Bio-Rad Laboratories, Alfred Nobel Drive Hercules, CA, USA) with SYBR Green intercalating dyes (Sintol, Moscow, Russia). The data were analyzed by the CFX Manager software (Bio-Rad Laboratories, Alfred Nobel Drive Hercules, CA, USA) with the $2^{-\Delta\Delta}$ Ct method [25]. Ubiquitin and tubulin genes were used as the reference genes. All the qRT-PCRs were run in three technical repeats. Primers used for qRT-PCR are

listed in Table S1. Dissociation curves (55–95 °C) were used to confirm the specificity of PCR amplification.

### *2.7. Statistical Methods and Computer Software*

Student's *t*-tests were used to compare gene expression levels.

To draw the box plots for nodule number in p35S::PsCLE12, p35S::PsCLE13, and control 35S::GUS plants RStudio (https://rstudio.com/, accessed on 11 July 2022) was used. A Mann–Whitney *U* test was used to compare nodule numbers in *MtCLE35*-overexpressing and control (GUS-overexpressing) plants; in each group from 10 to 17 plants were analyzed in each biological repeat. Three independent biological repeats were performed to analyze the effect of *PsCLE*s overexpression.

Multiple alignment of protein sequences was performed using UGENE software (http://ugene.net/ru/, accessed on 11 May 2022) [26] with Clustal W algorithm. Sequences were aligned using the MEGAX program (https://www.megasoftware.net/, accessed on 11 May 2022) with the Clustal W algorithm, and the phylogenetic tree was generated using maximum-likelihood method with 1000 bootstrap replicates.

## 3. Results

### *3.1. Nodulation-Related CLE Genes in Pea and their Expression Levels in Response to Rhizobial Inoculation and Nitrate Treatment*

In our previous study [19], we identified 45 transcripts of *CLE* genes in pea transcriptomic data [27]. Among them, *PsCam040153*, *PsCam040702*, *PsCam040984*, and *PsCam041632* sequences were closely related to nodulation-related *CLE* genes known from other legumes [19]. PsCam040153 and PsCam040702 were the closest homologues of *MtCLE12* and *MtCLE13*, respectively, which are the negative regulators of symbiosis [11]. Therefore, these genes were referred to as *PsCLE12* (PsCam040153) and *PsCLE13* (PsCam040702) [19].

The CLE domain of PsCLE13 appeared to be identical to the ones of LjCLE-RS1 and LjCLE-RS2 (see Figure 1), which inhibit nodulation in *L. japonicus* [10]. The CLE domains of PsCam040153 (which was referred to as the PsCLE12 [19]) has overall high sequence similarity with MtCLE12; however, it differs from the CLE domains of MtCLE12 in two positions (Figure 1). The CLE peptide corresponding to PsCam040984, previously referred to as PsCLE12-like [19], demonstrates high similarity with LjCLE-RS3 from *L. japonicus*: their two first amino acids are Trp and Ile but not Arg and Ile, as found in other nodulation-related CLE peptides. However, PsCLE12-like has the Asn residue at eighth position, similar to MtCLE12 and PsCLE12. Finally, at the very end of the CLE domain, the PsCLE12-like peptide has the Asn residue similar to the PsCLE12 and PsCLE13 peptides and their closest homologs from other legumes. This is in contrast to the CLE peptides from the NIC-like group, which have the His residue at end of their CLE domains (Figure 1). This group includes PsCam041632, which is referred to as *PsNIC*-like. The CLE domains of PsNIC-like is identical to MtCLE34 and closely related to nitrate-activated CLE peptides (PsNIC1-like) from soybean. The CLE proteins from the NIC-like group, including MtCLE34, PsNIC-like, and GmNICs, have sequence similarity not only within the CLE domains but also share the putative cleavage site sequences downstream of the signal peptide as well as demonstrate sequence similarity within the variable domain of the precursor proteins [28]. Interestingly, *MtCLE34* was found to be a pseudogene in *M. truncatula* A17 line but encodes a functional product in *M. truncatula* R108 line [28]. However, the *MtCLE34* gene from *M. truncatula* R108 line was unable to suppress nodulation [28]. The GmNICs from soybean were found to reduce nodulation acting locally in the root [9].

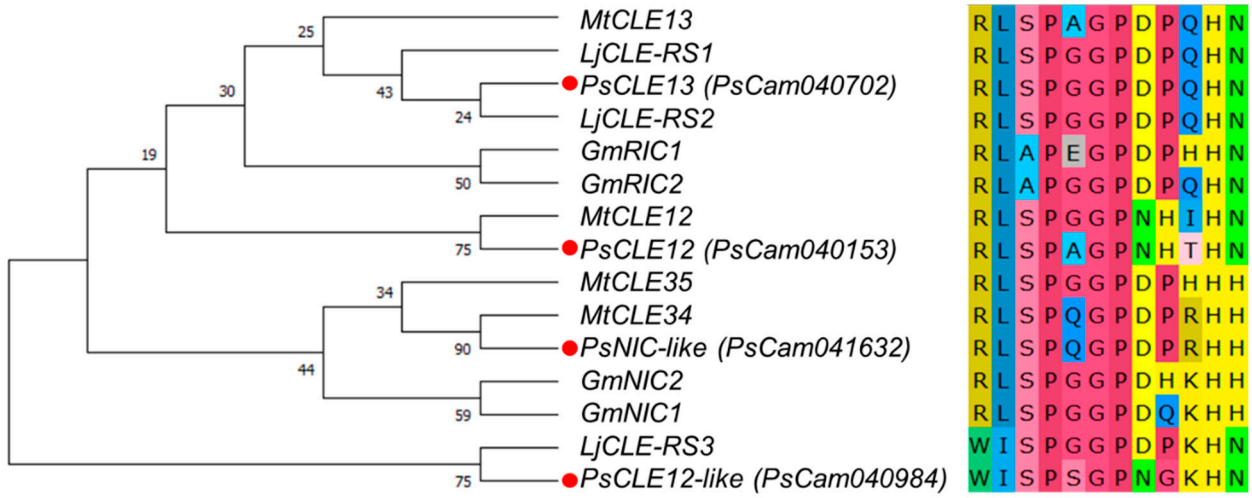


**Figure 1.** Nodulation-related CLE peptides in pea and other legumes. The phylogenetic tree was constructed in MEGA X based on amino acid sequences of the CLE domains using the maximum-likelihood method. Red dots mark nodulation-related *PsCLE* genes.

Previously, the expression levels of the *PsCLE12 (PsCam040153)*, *PsCLE13 (PsCam040702)*, PsCLE12-like (PsCam040984), and *PsNIC-like (PsCam041632)* genes were shown to be increased in the roots after rhizobial inoculation [19]. Data from this study confirmed the increased expression levels of these genes in developing nodules (Supplementary Figure S1A,B). Moreover, increased expression levels of the *PsCLE12, PsCLE13, PsCLE12*-like, and *PsNIC*-like genes in nodules were found in transcriptomic data obtained by Alves-Carvalho et al. [27] (Supplementary Figure S2).

Next, we checked the effect of nitrate treatment (10 mM $KNO_3$) on the expression levels of the *PsCLE* genes (Figure 2 and Figure S3). The addition of nitrate activated the expression of the *PsCLE12, PsCLE13,* and *PsNIC*-like genes after 24 h, and the expression levels of these genes remained increased after 48 of nitrate treatment (Figure 3).

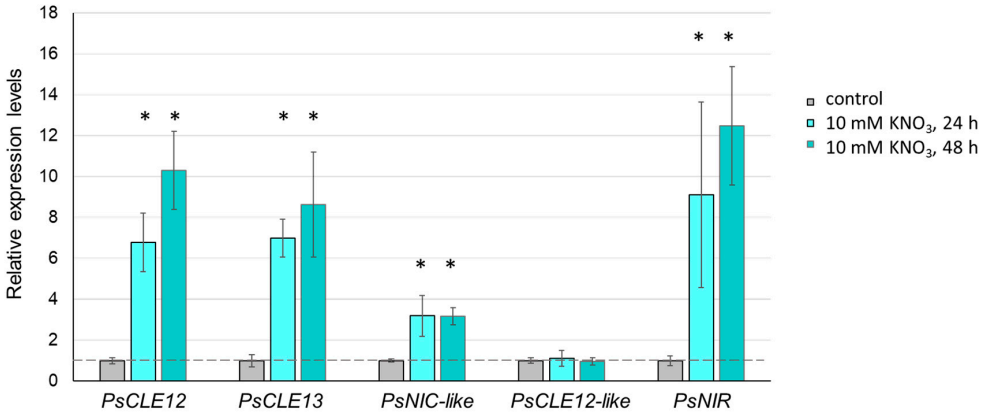


**Figure 2.** Expression levels of pea *CLE* genes in response to nitrate treatment. Results are means ±SEM of 4–5 biological repeats; Asterisks indicate statistically significant differences compared with the control (* $p < 0.05$) revealed by a Student's *t* test. The gene expression levels were normalized to 1 against the expression found in the control roots.

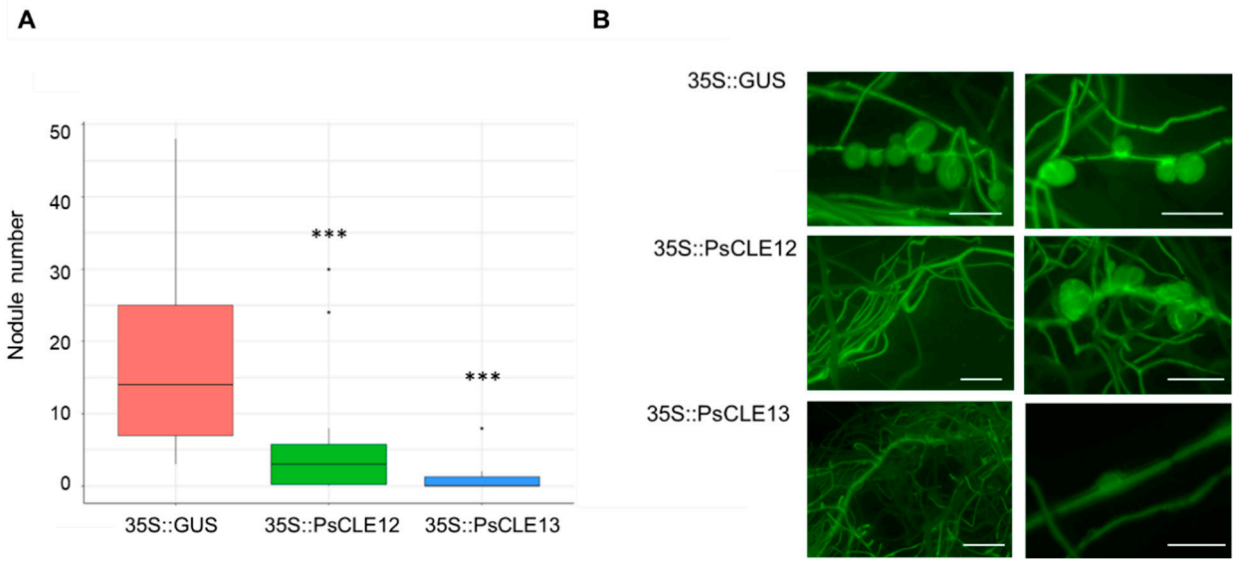


**Figure 3.** Overexpression of the *PsCLE12* and *PsCLE13* genes inhibits nodulation in pea. (**A**) Nodule number in GFP-positive transgenic roots transformed with the 35S:GUS (control), 35S:PsCLE12, and 35S::PsCLE13 constructs. Boxplots are shown, where center lines show the medians ($n$ = 15); box limits indicate the 25th and 75th percentiles. Asterisks indicate statistically significant differences (Student's $t$ test, *** $p < 0.001$). (**B**) Examples of the nodulation phenotype of GFP-positive transgenic roots carrying the 35S:GUS (control), 35S:PsCLE12, and 35S::PsCLE13 constructs. Bars = 100 µm.

However, the expression level of the *PsCLE12*-like gene was not influenced by nitrate addition (Figure 3 and Figure S3). Moreover, we analyzed the expression of pea nitrite reductase gene (PsNIR, Psat7g123960) [29], whose homologue in *Arabidopsis thaliana* (L.) Heynh. was characterized as a nitrate-regulated gene [30]. The expression of the *PsNIR* gene was also increased in response to nitrate in our experiments, suggesting the efficacy of nitrate treatment (Figure 3). Therefore, *PsCLE12, PsCLE13,* and *PsNIC*-like are induced by nitrate treatment, suggesting that these genes could be involved in nitrate-dependent control of nodulation.

### *3.2. Overexpression of the PsCLE12 and PsCLE13 Genes Suppresses Nodulation*

To find out the roles of two *PsCLE* genes, *PsCLE12* and *PsCLE13,* in nodulation, two constructs were obtained where the coding sequences of these genes were placed under the 35S promoter (35S:PsCLE12 and 35S::PsCLE13). These constructs were used for *A. rhizogene*-mediated transformation of pea, and as a result, composite plants with transgenic roots were obtained, which were selected based on fluorescence of GFP positive marker. As a control, GUS-overexpressing roots (35S:GUS) were used. Overexpression of *PsCLE12* and *PsCLE13* in transgenic roots was confirmed by qPCR analysis (Supplementary Figure S4).

Nodules formed on transgenic roots were counted at 28 dpi. The overexpression of both genes, *PsCLE12* and *PsCLE13*, resulted in a significant reduction of nodulation (Figure S3). *PsCLE13* was found to be a more potent inhibitor of nodulation since its overexpression inhibited nodulation almost completely, and only rare unmature nodules were found. The overexpression of the *PsCLE12* gene also significantly reduced nodule number on transgenic roots; however, the inhibitory effect was less pronounced in comparison with 35S::PsCLE13 roots, and mature nodules were still found on 35S::PsCLE12 roots (Figure 3).

### *3.3. 35S::PsCLE12 and 35S::PsCLE13 Transgenic Roots Demonstrate the Upregulation of Pea Homologues of TOO MUCH LOVE Genes*

Overexpression of nodulation-inhibiting *CLE* genes in *M. truncatula*, resulted in the upregulation of the *TML* transcripts in the root, which inhibited nodulation [14,17]. Here, we identified the homologues of *TML* genes in pea genome, *PsTML1* (Psat3g169800/PsCam036214)

and *PsTML2* (Psat1g087960/PsCam037480) and performed qPCR analysis of *PsTML1* and *PsTML2* expression in 35S:PsCLE12 and 35S::PsCLE13 transgenic roots. The expression levels of both *PsTML1* and *PsTML2* were significantly increased in *PsCLE13*- and *PsCLE12*-overexpressing roots (Figure 4 and Figure S5).

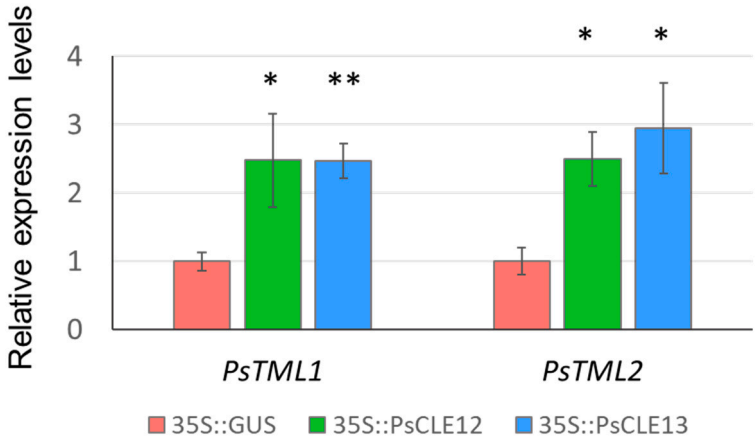


**Figure 4.** Expression levels of *PsTML1* and *PsTML2* genes in *PsCLE12*-overexpressing (35S::PsCLE12) and *PsCLE13*-overexpressing (35S::PsCLE13) transgenic roots. Asterisks indicate statistically significant differences (** $p < 0.01$; * $p < 0.05$) revealed by a Student's *t* test. Results are mean ± SEM of 3 biological repeats. The gene expression levels were normalized to 1 against the expression found in the 35S:GUS control roots.

Therefore, our data suggest that PsCLE13 and PsCLE12 could mediate inhibition of nodulation through the upregulation of *PsTML* genes in the root.

## 4. Discussion

Previously, we identified the transcripts of nodulation-related *CLE* genes in pea [19] found in transcriptomic data [28]. Moreover, the transcripts of the *PsCLE13*, *PsCLE12*, and *PsCLE12*-like genes were also identified in the assembly of the pea nodule transcriptome and were designated as *Cle13*, *Cle12a*, and *Cle12b*, respectively [31]. According to qPCR analysis, all these pea genes were activated in rhizobia-inoculated roots ([19] and Supplementary Figure S1).

Here, we found that *PsCLE12*, *PsCLE13*, and *PsNIC*-like genes were upregulated by nitrate addition, whereas *PsCLE12*-like was not responsive to the nitrate. In contrast to *PsCLE12* and *PsCLE13*, their close homologues in *M. truncatula*, *MtCLE12* and *MtCLE13*, *LjCLE-RS1* in *L. japonicas*, and *GmRIC1* and *GmRIC2* in soybean were not induced by the nitrate [9,11]. However, like *PsCLE12* and *PsCLE13*, the *MtCLE35* and *LjCLE-RS2* gene, which also belong to the same clade of *CLE*s, were activated by the nitrate [10,12–14]. Such differences in the ability of the *CLE* genes from different legumes to respond to the nitrate could be explained by distinct distribution of nitrate-responsive regulatory elements in the promoters of these genes. The ability of the *PsNIC*-like gene to respond to the nitrate places this gene together with all other genes of the NIC-like clade in legumes, which are nitrate-responsive [10,12,28]. Therefore, the *PsCLE12*, *PsCLE13*, and *PsNIC*-like genes could be possible regulators of symbiosis in response to nitrate in *P. sativum*.

For two genes, *PsCLE13* and *PsCLE12*, the effect of their overexpression on nodule number was analyzed, and both these genes inhibited nodulation in pea when overexpressed. The inhibitory effect of *PsCLE13* on nodulation was more pronounced and an almost complete block of nodulation was observed in all transgenic roots. However, the overexpression of *PsCLE12* did not result in the complete inhibition of nodule development in all the transgenic roots analyzed, and mature nodules were still observed on some 35S::PsCLE12 transgenic roots. This corresponds to the findings obtained for the homologues of these peptides in *M. truncatula*: the 12-amino-acid synthetic MtCLE13 peptide appeared to be far more potent AON-inducing peptide than MtCLE12 [32]. However, in

the study by Mortier et al. both the *MtCLE13* and *MtCLE12* genes inhibited nodulation completely when overexpressed [11]. In our previous study we found that *MtCLE13* overexpression also completely inhibited nodulation in pea, and this effect was shown to be dependent on the shoot-acting CLV1-LK kinase PsSYM29 [23]. Moreover, application of triarabinosylated synthetic GmRIC1 and GmRIC2 peptides also inhibited nodulation in pea in a PsSYM29-dependent manner [32]. The CLE peptides encoding by the *MtCLE13* and *GmRIC2* genes are almost identical to the one encoded by the *PsCLE13* gene (they differ only in one amino acid, see Figure 1), suggesting that the PsSYM29 receptor kinase could act as the receptor of endogenous CLE peptides from pea as well. However, a future study highlighting the effect of overexpression of pea *PsCLE* genes in the *sym29* mutant should provide direct evidence of involvement of the PsSYM29 receptor kinase in the reception of the CLE peptides encoded by the *PsCLE* genes.

Previously, it was found that overexpression of *MtCLE12*, *MtCLE13*, and *MtCLE35* genes in the root resulted in the increased expression levels of the *MtTML1* and *MtTML2* genes [14,17]. The *TOO MUCH LOVE1,2* transcripts are targeted by *miR2111*, a shoot-to-root transported miRNA, the amount of which is reduced due to the activation of nodulation-induced CLE signaling pathway in the shoot. Here, we showed that in the 35S::PsCLE13 and 35S::PsCLE12 transgenic roots the expression levels of *PsMTL1* and *PsTML2* were increased. Therefore, our data suggest that the PsCLE13 and PsCLE12 peptides trigger the accumulation of the *PsTML* transcripts in the root. This finding allows us to speculate that the conserved systemically acting miR2111-TML module could be involved in PsCLE12- and PsCLE13-mediated inhibition of nodulation. However, further studies with use of high throughput RNA and microRNA sequencing of transgenic pea roots overexpressing *PsCLE12* and *PsCLE13* are required to elucidate the targets of PsCLE13- and PsCLE12-mediated pathways inhibiting nodulation.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12112840/s1, Figure S1: Expression levels of pea *CLE* genes at different timepoints after inoculation with rhizobia; Figure S2: Heatmap representing expression levels of *PsCLE* genes in roots and nodules (according to the transcriptomic data by Alves-Carvalho et al., 2015); Figure S3: Expression levels of pea *CLE* genes in response to nitrate treatment: the results of the additional experiment. Figure S4: Expression levels of *PsCLE12* (A) and *PsCLE13* (B) in transgenic roots. Figure S5: Expression levels of *PsTML1* and *PsTML2* genes in *PsCLE12*-overexpressing (35S::PsCLE12) and *PsCLE13*-overexpressing (35S::PsCLE13) transgenic roots: the results of the additional experiment Table S1: List of primers used for qPCR analysis. Reference [33] is cited in the supplementary materials.

**Author Contributions:** Conceptualization, M.A.L. and L.A.L.; investigation, M.A.L., D.S.S. and D.A.D.; writing—original draft preparation, M.A.L., writing —review and editing L.A.L. and V.A.Z.; supervision, M.A.L., project administration, funding acquisition—V.A.Z. and L.A.L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This project was supported by the Russian Science Foundation project No. 21-66-00012 (overexpression study) and the grant from St. Petersburg State University ID: 93020341 (gene expression analysis).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Acknowledgments:** The authors thank the Research Resource Center for Molecular and Cell Technologies of Saint-Petersburg State University for the equipment used in this study and for the sequencing.

**Conflicts of Interest:** The authors declare no conflict of interest.

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