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Article

Nitrogen-Activated CLV3/ESR-Related 4 (CLE4) Regulates Shoot, Root and Stolon Growth in Potato

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Abstract: In potato, high levels of nitrogen (N) can lead to excessive vegetative growth at the expense of tuber development, resulting in lower yield and poor quality tubers. We found that *Solanum tuberosum* CLE4 (*StCLE4*) is expressed most strongly in the roots on N-rich media and positively regulates root growth of potato in N-deficient conditions. We noted that *StCLE4* functions as a negative regulator of normal shoot apex development similar to CLV3 in Arabidopsis. Transcriptomic analysis revealed that overexpression of *StCLE4* results in repression of the *StIT1* gene, a regulator of potato tuber initiation. Overexpressing *StCLE4* stolons were transformed into branches that were similar to milder *it1* phenotype. We also found that NIN-like proteins, key regulators of nitrate signaling, bind to the regulatory sequence of *StIT1* in yeast one-hybrid assay. Taken together, our findings suggest that *StCLE4* regulates shoot, root and stolon growth in potato.

Keywords: potato; CLE peptide; CLE4; NIN-LIKE PROTEINs (NLPs); nitrate; nitrate-responsive elements (NREs); shoot apical meristem (SAM); Identity of Tuber1 (IT1); tuber

1. Introduction

The CLAVATA3/EMBRYO SURROUNDING REGION (CLE) peptides are a group of hormones that play a crucial role in plant development and growth. In shoot apical meristem (SAM), CLV3 is expressed and regulates the size of the SAM by down-regulating expression of the WUSCHEL (WUS) gene encoding a homeodomain transcription factor. Other CLE genes, including CLE1, CLE4, CLE6, and CLE7 can complement the enlarged meristem of the *clv3* mutants when they are expressed under the control of the CLV3 promoter [1,2]. At the same time, the CLE1, CLE3, CLE4, and CLE7 genes are induced in roots under nitrogen-deficient conditions and regulate root growth and branching in response to nitrogen availability [3]. CLE genes have been identified in various plant species, including *Arabidopsis thaliana*, rice, and maize. Previously we have identified CLE genes in potato *Solanum tuberosum* L. (*StCLE*) and found that expression level of several CLE genes is regulated by nitrogen supply [4]. Nitrogen is a macronutrient essential for plant growth and development, and it is often a limiting factor in crop production. Adequate nitrogen supply is necessary for the formation of healthy and high-yielding potato tubers. During the early stages of tuber development, nitrogen plays a role in the formation of stolons, which are stem-like structures that develop from the lower nodes of the potato plant. As tuber development progresses, nitrogen is required for the accumulation of starch and other nutrients in the tubers. Nitrogen deficiency during this stage can result in reduced tuber size and lower yield. However, excessive nitrogen supply can also have negative effects on tuber development. Excessive nitrogen levels can cause an overabundance of vegetative growth, which can have a negative impact on tuber development, ultimately resulting in lower yields and lower quality tubers. Therefore, it is important to maintain a balanced nitrogen supply throughout the growing season to ensure optimal tuber development in potato plants.

We previously found that the *StCLE4* was induced in potato roots under N-rich growth conditions and suggested that *StCLE4* could be a negative regulator of tuberization. In this study, we show that the *StCLE4* promoter drives GUS reporter activity in the roots on N-rich media. Plants with

overexpression of *StCLE4* demonstrated a *wus*-like phenotype with arrested growth of the shoot apical meristem (SAM). Overexpression of *StCLE4* also resulted in stimulation of root growth and non-swelling stolons that were converted into branches. The transcriptomic studies of *StCLE4* overexpression plants revealed repression of the *IT1* gene, a regulator of potato tuber initiation. Overall, overexpression phenotype and RNA-Seq expression profiles suggest roles of *StCLE4* in regulation of shoot, stolon, and root growth.

2. Results

3.1. Promoter Activity of *StCLE4* Gene in Potato Roots

The *StCLE4* gene belongs to the same clade of CLEs as the nitrate-regulated CLE genes of *A. thaliana* (*AtCLE1*, -3, -4, and -7) [4] that are induced upon N deprivation and suppress the initiation of lateral roots [3]. Previously, we evaluated the expression of the *StCLE4* gene in the roots of potato exposed to different N-availability conditions using RT-qPCR. Opposite to *A. thaliana* CLE genes, the *StCLE4* gene was positively regulated by nitrogen supply [4]. In this study to determine the expression pattern of *StCLE4* in potato roots, we generated *promCLE4*:GUS fusion constructs using 2963 bp of the genomic region upstream of *StCLE4*. The *StCLE4* promoter drove GUS activity in the vascular tissues of the root (Figure 1b), and the reporter gene was expressed most strongly on N-rich media (Figure 1a), confirming that this gene is activated under high nitrogen conditions.

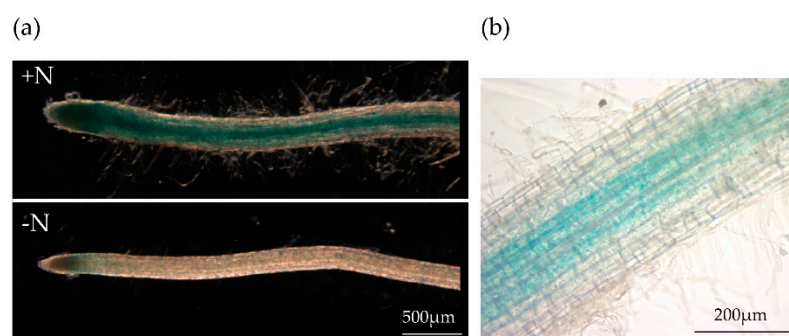


Figure 1. *promStCLE4*:GUS expression in potato roots. (a) The reporter gene was expressed most strongly on N-rich media (+N). (b) The *StCLE4* promoter activity in the vascular tissues of the root.

3.2. Effects of Overexpressing *StCLE4* and *StCLE4*^{G6T} Genes on Root, Shoot and Stolon Growth

To further investigate the functions of *StCLE4*, we used antagonistic peptide technology [5]. Specifically, we created an antagonistic *StCLE4* construct by mutating a codon encoding glycine in the CLE motif-coding region (which is known to play a crucial role in the biological activity of CLE) to threonine-coding codon (G6T) (Figure 2a). Vectors carrying the *StCLE4* and *StCLE4*^{G6T} genes under the constitutive 35S promoter were obtained. Overexpression of *StCLE4* resulted in stimulation of root growth in aeroponic nutrient medium without nitrogen (Figure 2bc) and reduction of tuber weight (Figure 2c). At the same time, we did not detect any alterations in growth of *StCLE4*^{G6T} overexpressing potato plants, except lower tuber weight (Figure 2, Figure S2).

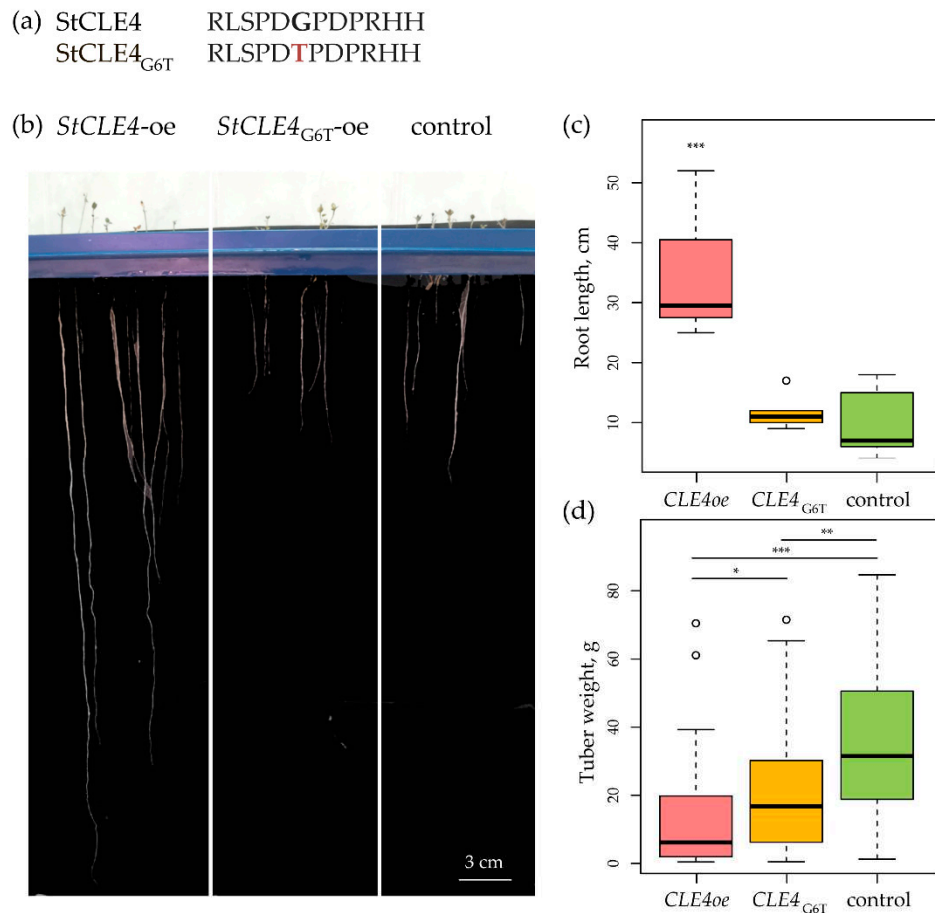


Figure 2. Native StCLE4 peptide and amino acid-substituted StCLE4_{G6T} peptide (a). Effects of StCLE4 and StCLE4_{G6T} overexpression on root growth in aeroponic nutrient medium without nitrogen (b) and tuber weight (c). *, p-value <0.05; **, p-value <0.01; ***, p-value <0.001.

Overexpression of StCLE4 also demonstrated phenotype similar to the phenotypes described for the overexpression of CLV3, CLE2, -3, -4, -5, -6, -7, -9, -10, -11, and -13 genes in *A. thaliana* [6]. The observed phenotypes were characterized by arrest of leaf development from the SAM and subsequent recovery via the activation of axillary buds (Figure 3a), which is very similar to *wus* loss-of-function mutants [7]. In contrast to *Arabidopsis* plants overexpressing CLV3, CLE9, -10, -11, and -13, all potato plants overexpressing StCLE4 resumed growth after the initial SAM arrest (Figure 3a). Also non-swelling stolons that converted into aerial shoot branches were observed on plants overexpressing StCLE4 (Figure 3b, Figure S2).



Figure 3. Effects of overexpressing StCLE4 (StCLE4-oe) gene on apex growth of three weeks old plants (a) and stolon growth of two months old plants (b) that were grown in growth chamber under long days (16 h light:8 h dark and 22°C). Non-swelling stolons that transformed into branches marked by red asterisk. Scale bar in panel (a) = 2 mm.

3.3. RNA-Sequencing of Transgenic Potato Leaves and Roots with StCLE4 and StCLE4_{G6T} Overexpression

To study the effect of gene overexpression on the potato transcriptome, we performed RNA sequencing analysis of leaves and roots of transgenic potato plants overexpressing the StCLE4 and StCLE4_{G6T} genes. RNA was isolated from both nitrogen-rich and nitrogen-free media grown plants. The obtained RNA-seq data were analyzed using bioinformatics tools to identify differentially expressed genes (DEGs) between transgenic and control plants. Gene ontology analysis was performed to identify the biological processes affected by StCLE4 and StCLE4_{G6T} overexpression. The results showed that the overexpression of the StCLE4 and StCLE4_{G6T} genes altered the expression of genes involved in regulation of shoot development, response to gibberellin, leaf development, auxin homeostasis and other processes (Table S2). Based on our RNA-Seq data we supposed that long-root phenotype can be caused by altering expression of auxin response genes, including SMALL AUXIN-UP RNAs (SAURs), Glycoside hydrolase 3 (GH3) and auxin response factor (Table S2). Among DEGs in StCLE4 overexpressing leaves, the Identity of Tuber 1 (StIT1) gene was found. StIT1 encodes a TCP transcription factor that determines tuber identity and interacts with the tuberization signal SP6A. The stolons of it1 knockout mutants were converted into aerial shoot branches, instead of swelling [8]. Based on our RNA-Seq analysis and the observed effects of StCLE4 overexpression it could be speculated that the StCLE4 peptide may initiate a signaling pathway that results in the inhibition of the StIT1 gene expression.

3.4. Yeast one-hybrid assay of interaction between StNLPs and the promoters of the StCLE4, StBEL5, StSP6A, and StIT1 genes

We next performed yeast one-hybrid (Y1H) assay to identify putative regulators of StCLE4. Previously we found, that crucial regulator of the photoperiod-dependent tuberization StBEL5 did not bind to DNA sequence corresponding to 500 bp upstream of the start codon of the StCLE4 gene

[4]. In this study we performed analysis of direct binding of key nitrate regulators NLPs to the promoter regions of *StCLE4*. NIN-like proteins (NLPs) are a family of transcription factors that play important roles in plant growth and development, as well as in plant-microbe interactions. The name "NIN-like" comes from the fact that these proteins are structurally similar to the Nodule Inception (NIN) protein, which is involved in the formation of root nodules in legume plants. One of the key functions of NLPs is the regulation of nitrate signaling and metabolism [9,10]. In *Arabidopsis*, the AtNLP6 and AtNLP7 proteins are crucial in regulating the expression of genes that respond to nitrate [9–11]. Using NLP protein sequences from *A. thaliana*, *Medicago truncatula*, and *Solanum lycopersicum* as queries, three *StNLP* genes homologues to AtNLP6 and AtNLP7 were identified (*StNLP3.1* (Soltu.DM.08G004830.1), *StNLP3.2* (Soltu.DM.08G004830.2) and *StNLP5* (Soltu.DM.08G029670.1)) (Figure S3). Among two variants of *StNLP3* gene, *StNLP3.1* was selected for further investigation due to its higher expression levels in compared to *StNLP3.2* (Table S2) (means of transcripts per million (TPM) values are 1722 and 80, respectively). The *StNLP5* gene is also highly expressed in all samples (mean of TPM = 2037).

NLPs are known to bind to nitrate-response elements (NREs) and activate the expression of nitrate-regulated genes in response to high nitrate levels [11]. We searched for the NREs in the promoters of the *StCLE4* gene, and in promoters of major regulators of tuber development (*StBEL5*, *StSP6A*, and *StIT1* genes) using the motifs described in the studies by Nishida et al. [12] and Laffont et al. [13] In the promoters of the *StIT1*, *StCLE4*, and *StBEL5* genes we found one, two and three putative NLP-binding sites, respectively (Figure 4a, Table S3). Next, we checked whether these putative NREs motifs could be bound by *StNLP3.1* and *StNLP5* using yeast one-hybrid assay. We found that *StNLP3.1* and *StNLP5* bound to the promoter of the *StIT1* gene whereas no interaction was detected for the *StCLE4* and *StBEL5* promoters (Figure 4b).

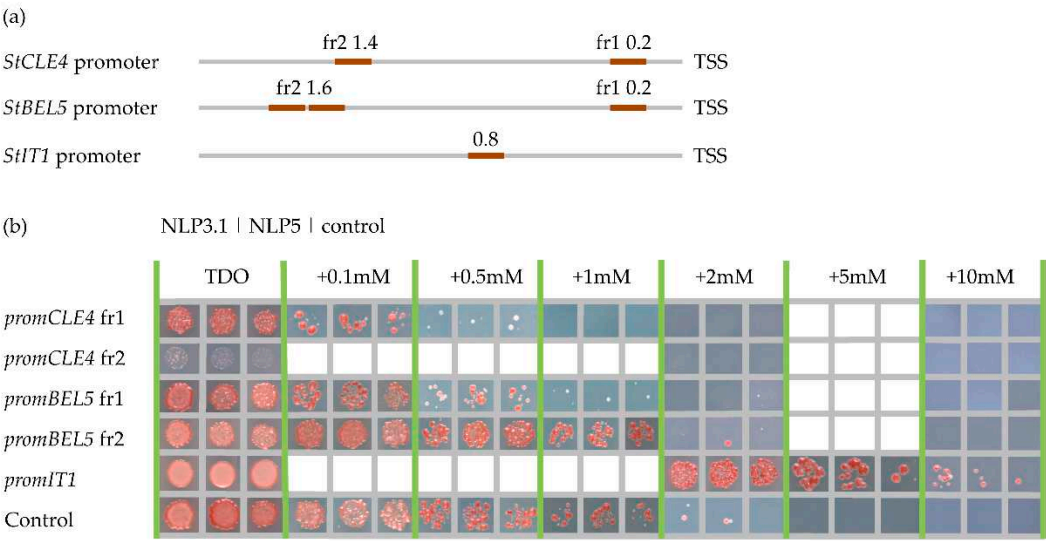


Figure 4. Interaction of *StNLP3.1* and *StNLP5* with *StCLE4*, *StBEL5*, and *StIT1* promoters (a) Approximate locations of the NRE motifs (according to Nishida et al. [12] and Laffont et al. [13]) in *StCLE4*, *StBEL5*, and *StIT1* promoters. The numbers indicate the distance from the translational start site (TSS). (b) Yeast one-hybrid assays of the interaction of *StNLP3.1* and *StNLP5* with *StCLE4*, *StBEL5*, and *StIT1* promoters. Controls: empty pHISLEU or pDEST22 vectors.

3. Discussion

Nitrogen is a crucial nutrient for the growth and development of potato plants, including tuber development. Previously, we found that the *StCLE4* gene was positively regulated by nitrogen supply and suggested that *StCLE4* could be a negative regulator of tuberization. In this study we found that the *StCLE4* overexpressing plants demonstrated *wus*-like shoots with arrested growth of the SAM and subsequent recovery of shoot growth via the activation of axillary buds. This phenotype was similar to that described for overexpression of *CLV3*, *CLE2*, -3, -4, -5, -6, -7, -9, -10, -11, and -13

genes in *A. thaliana* [6]. Like Arabidopsis plants with *AtCLE1-7* overexpression, potato plants overexpressing the *StCLE4* gene resumed growth after the initial SAM arrest. Furthermore, the *StCLE4* is close to these *AtCLE* proteins on phylogenetic tree [4]. Besides this, *StCLE4* overexpression promoted root growth, and similar effect was also observed for *AtCLE2*, *AtCLE4-7* overexpression [6]. However, opposite to *A. thaliana* *CLE1*, -3, -4, and -7 genes, the *StCLE4* gene was positively regulated by nitrogen supply, like the *AtCLE2* and *AtCLE6* genes [3]. At the same time *AtCLE2* and *AtCLE6* promoters drive specific GUS staining in the cells at the junction between the primary root and the lateral roots [14], whereas *StCLE4* reporter expression was detected in the vascular tissues of the root. Therefore, *StCLE4* is closely related to Arabidopsis *AtCLE1-7* peptides according to phylogenetic analysis and functional studies; however, regulation of *StCLE4* activity in potato is different from that described for the *AtCLE1-7* genes.

Overexpression of *StCLE4* not only affected shoot apex and root growth but also promoted stolon conversion into branches. We performed RNA-Seq analysis of leaves and roots of transgenic and control plants. At the same time downregulation of the *Identity of Tuber 1 (StIT1)* gene was found in the leaves of the *StCLE4* overexpressing plants grown in N-deficient media. *IT1* is a transcription factor that directly interacts with mobile tuberigen, SP6A, and regulates tuber identity [8]. The stolons of *it1* knockout mutants were converted into branches, instead of swelling [8]. Opposite to *it1* mutants, *StCLE4* overexpressing plants produced tubers, but tuber weight of these tubers was lower than controls. Therefore, we suppose that *StCLE4* promotes growth of stolons as shoots, similar to milder *it1* phenotype, and that *StCLE4* overexpressing phenotype could be explained by downregulation of *IT1*, that in turn regulates stolon swelling.

The NLPs transcription factors can respond to nitrate signaling, regulate gene expression, and participate in N absorption and utilization. Previously, it was shown that NLP can regulate the expression of the *CLE* genes [13,15,16], and therefore we assumed that *StNLP3* and *StNLP5* can bind to the promoter region of *StCLE4*. However, we did not detect this interaction in yeast one-hybrid system. Moreover, previously, we also did not find interaction of photoperiod-dependent tuberization factor *StBEL5* and the promoter region of the *StCLE4* gene [4], and therefore, we still do not know which transcription factor regulates the *StCLE4* gene expression. However, we found that *StNLP3* and *StNLP5* bound to the promoter of *StIT1*. Previously, it was shown that N plays an important role in regulating tuberization [17], and based on our data, we speculate that the *StCLE4* peptide, as well as the *StNLP3* and *StNLP5* transcription factors, may act on N-dependent tuberization pathway and regulate the expression of *StIT1*, however, further investigations are necessary to study the mechanisms of such regulation in more detail. Further research on the mechanisms of *CLE* genes regulation and their interactions with nitrogen signaling pathways will be crucial for understanding how plants respond to changes in nitrogen availability and for developing strategies to improve crop productivity.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Tubers of the *Solanum tuberosum* cultivar Desirée was obtained from the stock of N. I. Vavilov All-Russian Institute of Plant Genetic Resources (Saint-Petersburg, Russia). The plants were propagated by two-node stem cuttings and were cultivated *in vitro* on a Murashige and Skoog (MS) medium containing 0.8% (w/v) agar and 1% sucrose under long-day conditions (16 h light:8 h dark) at 22 °C. N-depleted or N-rich medium was prepared as described in [18]. For investigation of effects of *StCLE4* and *StCLE4_{G6T}* overexpression on root growth, aeroponic nutrient medium without nitrate was used [19]. Seven days old stem cuttings were transferred into aeroponic system in a controlled greenhouse (16 h light:8 h dark and 22 °C) and were grown for four weeks. The root length was measured using the ImageJ [20] software, and a Tukey's test was used to compare the means of three groups. Stem cuttings were also transferred to the pots (9x9x9 cm) with vermiculite and grown in growth chamber for three months (16 h light:8 h dark and 22°C first two months; 8 h light:16 h dark and 22 °C for induction of tuber development). Twice a month plants were supplied with ¼ MS media

without sucrose. Tubers, obtained from plants growing in the vermiculite, were planted in the field (Leningrad Region, Russia) in June 2023 and were grown for 2.5 months. Tuber weight of plants grown in the field was measured, and a Tukey's test was used to compare the means of three groups.

4.2. Constructs

The primers used for PCR are listed in Table S2. Polymerase chain reaction (PCR) with Phusion High-Fidelity DNA Polymerase (Thermo Scientific, USA) was used to amplify coding sequences or predicted promoter fragments of genes from whole plant DNA of *Solanum tuberosum* L. cv Desiree. PCR fragments were extracted from an agarose gel using a Cleanup Mini Kit (Evrogen, Russia) and cloned into the entry vector pDONR207. Then, coding sequences were cloned into the vector pMDC32 [21], under the CaMV 35S promoter, via Gateway technology, according to the manufacturer's instructions (Invitrogen, USA). Promoter sequences for GUS or GFP reporter analysis were cloned into the vector pBGWFS7 [22] or pHm43GW (Invitrogen, USA). For yeast one-hybrid assay, the promoter fragments of the *StCLE4*, *StSP6A*, and *StIT1* genes (Table S1) were cloned into the pHISLEU2GW vector (kindly provided by Dr. Rogers from Cardiff University). Coding sequences of *StNLP3.1* and *StNLP5* were introduced into pDEST22 (Invitrogen, USA). The resulting plasmids were transformed into Top10 *Escherichia coli* Competent Cells. Plasmid DNA was isolated using Plasmid Miniprep Kit (Evrogen, Russia). Each plasmid was checked by sequencing. The resulting binary vectors were transferred to the *Agrobacterium rhizogenes* strain Arqua or *Agrobacterium tumefaciens* strain AGL1. *Agrobacterium* cultures were grown overnight on a shaking incubator at 28–30°C in Luria-Bertani supplemented with antibiotics.

4.3. Transformation of potato

For hairy potato root transformation with the promStCLE4::GUS construct, stems were wounded and inoculated with the *A. rhizogenes* and then co-cultivated on Murashige and Skoog (MS) media with 10 g/L sucrose for 2 days. Transgenic hairy roots were grown in MS medium supplemented with cefotaxime (500 mg/L) to prevent *Agrobacterium* overgrowth.

Transgenic *S. tuberosum* plants containing prom35S::StCLE4, prom35S::CLE4G6T constructs were generated by *Agrobacterium tumefaciens*-mediated transformation mainly as described previously [23–25]. The wounded leaf explants were incubated with agrobacteria in liquid MS medium with 16 g/L glucose for 2 days. Then the explants were moved to solidified MS medium (8 g/L agar) that induces callus growth (16 g/L glucose; 500mg/L cefotaxime; 3 mg/L hygromycin; 5 mg/L NAA; 0.1 mg/L BAP) and were incubated for 7–8 d under LD at 27°C. The explants were later transferred to a shoot regeneration MS medium (16 g/L glucose; 500mg/L cefotaxime; 3 mg/L hygromycin; 1 mg/L BAP; 0.1 mg/L GA) and were repeatedly transferred to fresh medium with the same composition every 10 days until shoots developed on the calli. The resulting 3 cm shoots were then transferred to MS medium (10 g/L sucrose; 500mg/L cefotaxime) for rooting.

4.4. Histochemical Assays

β-Glucuronidase (GUS) staining was performed as described [26]. Incubation periods ranged from 2 to 16 h following vacuum infiltration. Roots were imaged using a ZEISS SteREO Discovery.V12 microscope.

4.5. RNA Isolation and RNA-Seq

For RNA-Seq analyses, total RNA was extracted from samples using the RNeasy Plant Mini Kit (Qiagen, USA). Enrichment of mRNA was performed using NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, USA). The libraries were prepared using NEBNext® Ultra™ II Directional (New England Biolabs, USA). For sequencing HiSeq PE Rapid Cluster Kit v2 and HiSeq Rapid SBS Kit v2 was used. Manufacturer's instructions were used for all procedures. The libraries were sequenced on the Illumina HiSeq 2500 sequencing platform.

Reads quality control was performed with FASTQC v. 0.11.9 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). FastQC reports were aggregated by MultiQC v. 1.14 [27]. The trimmomatic v. 0.39 program [28] was used to trim reads from technical artifacts. HISAT2 v. 2.2.1 [29] was used as alignment program for mapping reads to a doubled monoplod potato DM 1-3 516 R44 genome v. 6.1. BAM files were sorted by samtools v. 1.13. Quantitation of RNA-Seq data was performed by StringTie v. 2.2.1 [30]. The *StCLE* and *StCEP* (C-terminally encoded peptide) [31] genes that have not been annotated previously as coding sequences were manually included in annotation. Differential gene expression analysis was performed using DeSeq2 v. 1.40.2 [32] in R.

2.6. Yeast One-Hybrid Assay

The 2.5-kb sequences upstream of start-codon were used for the promoter analysis. Putative NRE motifs were identified using sequences revised in several recent studies [12,13] and MAST algorithms available on the MEME suite server (version 5.1.0, <http://alternate.meme-suite.org/tools/meme>). Transformation of *Saccharomyces cerevisiae* strain Y2HGold (Clontech) was performed as described in [33]. A yeast one-hybrid assay was performed as described in [34].

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: The phenotype of *StCLE4_{G6T}* overexpressing plants that were grown in growth chamber; Figure S2: The phenotype of *StCLE4* (*StCLE4-oe*) and *StCLE4_{G6T}* (*StCLE4_{G6T}-oe*) overexpressing plants, and control plants that were grown in the field; Figure S3: Phylogenetic tree of *Arabidopsis thaliana*, *Medicago truncatula*, *Solanum lycopersicum*, *Oryza sativa*, *Lotus japonicas*, and *Solanum tuberosum* NLP protein families; Table S1: Differentially expressed genes (DEGs) in leaves and roots from nitrogen-rich media grown control and transgenic plants; Table S2: List of primers; Table S3: Potential NRE sites in promoter regions of *StCLE4*, *StBEL5*, *StIT1*, which were used for yeast-one-hybrid analyses.

Author Contributions: Conceptualization, L.L. and M.G.; methodology, M.G.; validation, M.G.; investigation, M.G.; resources, L.L. and M.G.; writing, M.G.; funding acquisition, M.G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All sequence reads of the RNA-Seq analysis have been deposited at the National Center for Biotechnology Information database under BioProject accession number PRJNA1007918.

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

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