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Phloem-Expressed *CLAVATA3/ESR*-like Genes in Potato

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Abstract: In potato, phloem tissues transport sugars and signal molecules to the tuber for growth and storage. The CLAVATA3/ESR-like (CLE) family of plant peptides plays an important role in regulating plant development. In this study, we identified a set of phloem-expressed *CLE* genes in *Solanum tuberosum* L. (*StCLE*s). We analyzed the phloem transcriptome of potato and found that 10 out of 41 *StCLE* genes were expressed in phloem cells, with *StCLE12* and *StCLE19* showing the highest expression levels. StCLE12 has an identical CLE domain to the Arabidopsis TDIF peptides, which are known to play a crucial role in maintaining the vascular meristem. StCLE19 has the highest sequence similarity to the Arabidopsis CLE25 peptide, which is involved in the formation of the phloem element and signaling in response to dehydration stress. The overexpression of *StCLE12* and another potato *TDIF*-like gene, *StCLE8*, promoted vascular cell proliferation and delayed leaf senescence. On the other hand, plants with overexpression of *StCLE19* were unable to form adventitious roots and demonstrated the absence of ordered cambium cell layers in the vascular bundles.

Keywords: potato; CLE peptides; TDIF; CLE25; phloem

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1. Introduction

Tuberization in potato (*Solanum tuberosum* L.) is a complex process that starts with the formation of an underground shoot called a stolon. During tuber initiation, several changes occur in the stolon tips. First, cell divisions are observed in the subapical region of the nonswelling stolons. Second, new conductive tissues appear to transport water and nutrients to a new storage reserve. As a result, a massive tuber, accumulating large amounts of starch, is formed. In plants, phloem tissues transport sugars and signal molecules that sink organs to regulate their growth and the storage of nutrients.

CLAVATA3/ESR-like (CLE) are peptide hormones that play a central role in plant development, as well as the formation of phloem. Each *CLE* gene encodes a pre-propeptide that includes an N-terminal signal sequence, guiding the protein through the secretory pathway as a variable region in the middle and a highly conserved 12–13 amino acid CLE domain in the C-terminus. The mature CLE is processed from larger pre-propeptides into a 12–13 amino acid peptide corresponding to the CLE domain sequence. The *Arabidopsis* genome contains 33 *CLE* genes that encode 28 unique peptides [1,2]. The *CLE25*, *CLE26*, *CLE33*, *CLE41*, *CLE44*, and *CLE45* genes are specifically expressed in the phloem, and developmental roles for them are known or suggested. The CLE25 peptide positively regulates phloem initiation [3], in contrast to the CLE45 peptide, which suppresses sieve element differentiation [4,5]. The CLE33 peptide acts redundantly with CLE45 [2], and the CLE26 peptide also behaves similarly to CLE45 but has additional signaling pathways that regulate root development [6]. CLE41 and CLE44, which are known as tracheary element differentiation inhibitory factor (TDIF) peptides, are also produced in the phloem cells and are transported to the vascular cambium, where they stimulate the proliferation of stem cells and inhibit the differentiation of xylem elements [7].

Although phloem plays a crucial role in the efficient functioning of potato tubers as storage organs, the genetic mechanisms of phloem formation in potato have not been

previously explored. Here, we identified and analyzed the effect of overexpression in three phloem-expressed potato genes, *StCLE12* and *StCLE8*, which encode TDIFs, and *StCLE19*, which is close to AtCLE25. We demonstrated the impact of these *CLE* genes on cambium activity and vascular system formation and also on the overall development of potato plants. The transcriptomic analysis of transgenic potato stems with the overexpression of *StCLE12* and *StCLE19* enabled us to hypothesize about the genetic mechanisms underlying the observed phenotypic changes. This includes speculating about the potential new targets of CLE peptides from two distinct groups.

2. Materials and Methods

2.1. Plant Material and Plant Growth Conditions

Tubers of the *S. tuberosum* cultivar Desirée were obtained from the N. I. Vavilov All-Russian Institute of Plant Genetic Resources (Saint-Petersburg, Russia). The plants were grown 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.

The transgenic *S. tuberosum* plants were generated using *Agrobacterium tumefaciens*-mediated transformation, as described previously [8].

Two-node stem cuttings from the control and transgenic plants were rooted in vitro on an MS medium and were transferred to the pots (9 × 9 × 9 cm) with vermiculite, where they were grown 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 the induction of tuber development). Once a week, plants were supplied with 1/4 of the MS media without sucrose. Tuber weight was measured, and Student's *t*-test was used to compare the means of the two groups.

For in vitro tuberization, one-node cuttings were transferred to the MS media with 8% sucrose, where they were grown for 8 weeks under dark photoperiod.

2.2. Genetic Constructs

The coding and promoter regions of *StCLE* were amplified using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, MA, USA) with the primers listed in Table S1. PCR products were extracted from agarose gel using a Cleanup Mini Kit (Evrogen, Moscow, Russia), cloned into the pDONR207 vector using the Gateway BP reaction, and transformed into the Escherichia coli strain DH10B. Next, the coding sequences of the *StCLE8*, *StCLE12*, and *StCLE19* genes were transferred into the pMDC32 vector [9] under the CaMV 35S promoter using the Gateway LR reaction. The 5′ upstream regions of the *StCLE19* and *StCLE8* genes were cloned into either the pBGWFS7 vector [10] for GUS reporter analysis or the pHm43GW vector (Invitrogen, Waltham, MA, USA) for GFP reporter analysis. The LR reaction products were then transformed into DH10B, and the resulting binary vectors were transferred into the *A. tumefaciens* strain AGL1.

2.3. Analysis of Coding and Promoter Sequences of the StCLE19 Gene

To identify the coding and promoter regions of *StCLE19* in *Solanum* genomes, BLASTN analyses with the *StCLE19* gene and promoter from the reference genome (*S. tuberosum* group Phureja DM1-3 516 R44) as a query were used against the genome assemblies of various potato species (*S. commersonii* GCA_001239805.1, *S. verrucosum* GCA_900185145.1, *S. bukasovii* GCA_009849815.1, *S. chaucha* GCA_009849625.1, *S. juzepczukii* GCA_009849685.1, *S. curtilobum* GCA_009849645.1, *S. ajanhuiri* GCA_009849805.1) and tomato (*S. lycopersicum* GCA_000188115.4) was available in the NCBI database. The alignment of nucleotide sequences was generated using MEGA7 software alongside the muscle algorithm [11] and was visualized using the Geneious Prime 2023 software (https://www.geneious.com/, accessed on 1 January 2023).

2.4. RNA-Seq and Data Analysis

Stems were collected from three two-week-old plants, which were grown in vitro, frozen in liquid nitrogen, and stored at −80 °C. The total RNA was isolated using the

RNeasy Plant Mini Kit (Qiagen, MD, USA). The enrichment of mRNA was conducted using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Hitchin, UK). The RNA-seq experiments were conducted using NEBNext® Ultra™ II Directional (New England Biolabs, USA), the HiSeq PE Rapid Cluster Kit v2, and the HiSeq Rapid SBS Kit v2. The manufacturer's instructions were used for all procedures. The libraries were sequenced on the Illumina HiSeq 2500 sequencing platform.

Quality control was performed with FASTQC (v. 0.11.5) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 1 July 2022). The trimmomatic v. 0.39 program [12] was used to trim reads from technical artifacts. Mapping reads to a potato genome v. 6.1 (DM 1-3 516 R44) was performed using HISAT2 v. 2.2.1 [13]. BAM files were sorted using samtools v. 1.13. Transcriptome assembly was carried out by StringTie v. 2.2.1 [14]. The *StCLE* genes that had not been annotated previously as coding sequences were manually included in the annotation. The differential analysis of count data was performed using DeSeq2 v. 1.40.2 [15] in R. The TPM (transcripts per kilobase million) values of phloem-expressed genes were counted using kallisto [16].

2.5. Histochemical Assays

The Leica VT1200S vibrating blade microtome was used to section the potato tubers. GFP localization was analyzed using the fluorescence microscope, Leica DM4000. Fluorescence and brightfield images were merged using ImageJ v. 1.53k [17] software. β-Glucuronidase (GUS) staining solution (0.05M sodium phosphate buffer, 5mM potassium ferricyanide, 1mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronide) was used until the stain was visible following vacuum infiltration. The reaction was performed at 37 °C and was stopped by 70% ethanol. Stained plants were imaged using a ZEISS SteREO Discovery.V12 microscope (Zeiss, Oberkochen, Germany).

3. Results

3.1. Screening for CLE Genes Expressed in the Phloem

To identify phloem-expressed *CLE* genes, we first obtained Illumina RNA-Seq data from the NCBI database. In Lin et al. [18], laser capture microdissection was used to isolate the phloem tissue of the petiole and the lower stem of short-day-grown potato plants for further RNA extraction and sequencing. We surveyed the TPM value of 41 *StCLE* genes in the phloem in the potato stem and found that 10 of them were expressed in the phloem cells (Table S2). *StCLE12* and *StCLE19* showed the highest expression levels (Figure 1a).

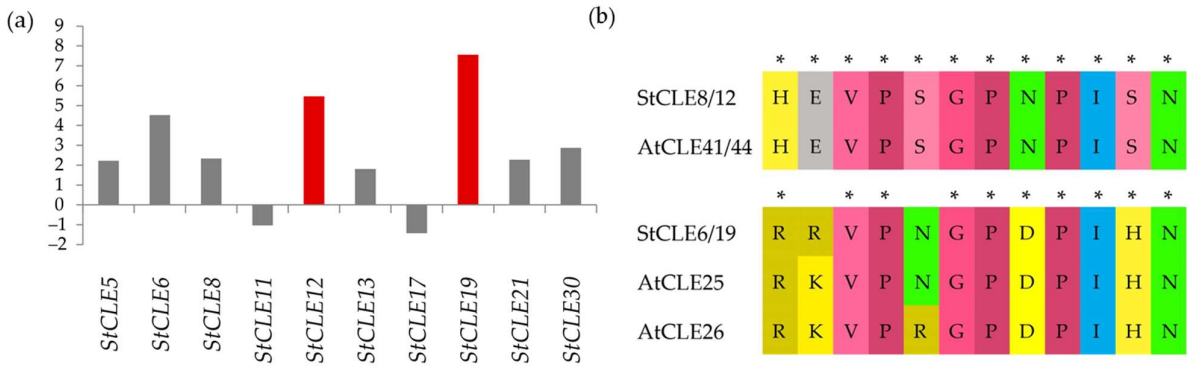

Figure 1. (**a**) Log2 TPM values of the *StCLE* genes in potato phloem. Only genes with non-zero TPM values are shown in the figure. (**b**) Alignment of the CLE peptide sequences found in the StCLE8/12, StCLE6/19, AtCLE41/44, AtCLE25, and AtCLE26 pre-propeptides. Conserved amino acids are indicated by asterisks. The default coloring for amino acid residues in the Ugene [19] is used.

We then examined the phylogenetic relationship of the *StCLE12* and *StCLE19* genes to *AtCLE*. We found that StCLE12 had an identical CLE domain to the TDIF peptide encoded by AtCLE41 and AtCLE44 (Figure 1b). *TDIF*s are expressed in the phloem cells and play

a crucial role in vascular cambium maintenance [7]. An identical TDIF peptide sequence has been reported across many species [20–22]. We established that StCLE19 shared the highest sequence similarity with AtCLE25, except that lysine in AtCLE25 was substituted by arginine in StCLE19 at position 2 (Figure 1b). AtCLE25 is a phloem-specific protein that is necessary for sieve element formation [3] and long-distance signaling in the dehydration stress response [23]. We also noted that StCLE8 and StCLE6 shared identical CLE domains with StCLE12 and StCLE19, respectively (Figure 1b).

3.2. Effects of Overexpression of StCLE12 and StCLE19 Genes on Root, Shoot, and Tuber Growth

StCLE12-overexpressing (*StCLE12*-oe) potato plants had an increasing number of cambium cell layers in the stem and lacked lignified parenchyma in the vascular bundles (Figure 2a,c). Transgenic plants exhibited a dwarf phenotype with small, dark green leaves (Figure S1). Furthermore, *StCLE12*-oe plants died due to stem damage associated with the dramatic activation of cambium cell divisions (Figures 2g and S1); thus, no adult plants were obtained. At the same time, we obtained *StCLE8*-oe plants, which demonstrated similarities to *StCLE12*-oe but a less pronounced phenotype (Figures 2b,f and 3a–d). Adult *StCLE8*-oe plants showed a bushy phenotype with the promotion of axillary bud outgrowth, aerial tuber formation (Figure 3e,f), a delay in leaf senescence (Figure S2), and a reduction in tuber weight (Figure 3g).

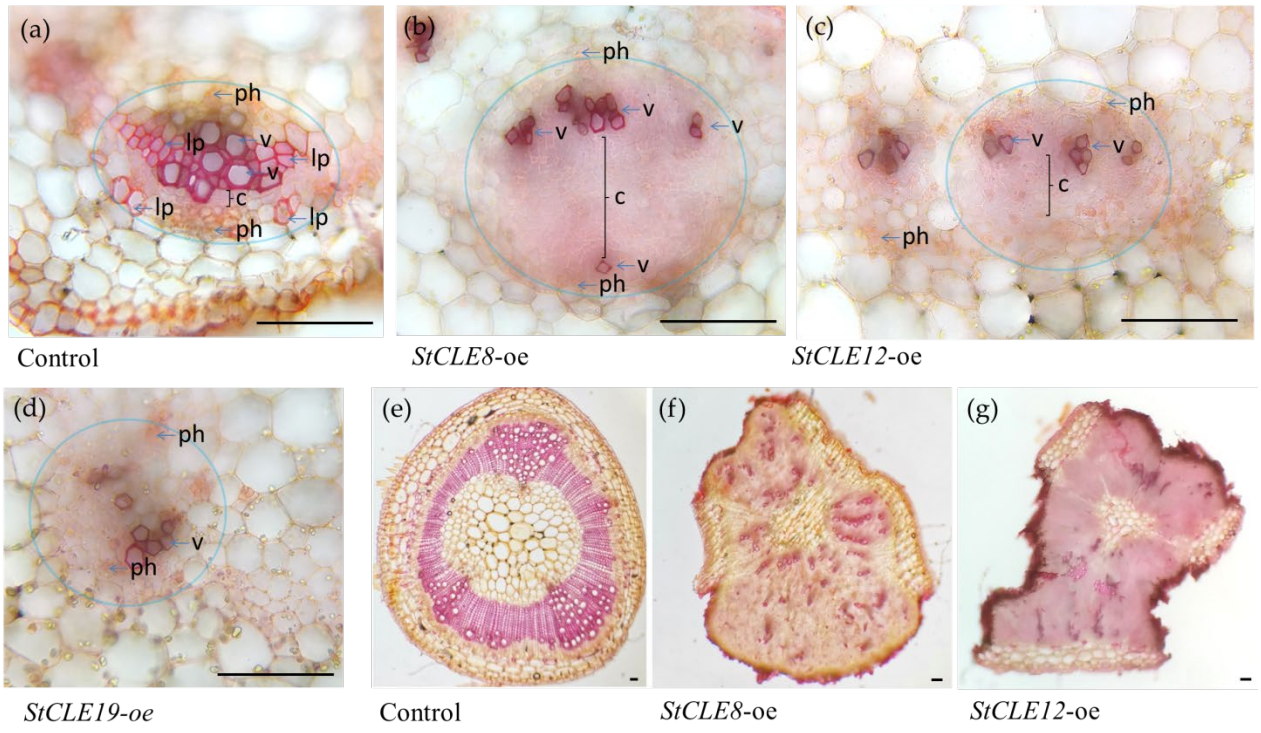

Figure 2. Effects of *StCLE* overexpression on the development of potato stems. (**a**–**d**) Transverse sections of vascular bundles of the two-week-old control plant (**a**), *StCLE8*- (**b**), *StCLE12*- (**c**), and *StCLE19*- (**d**) overexpressing plants. (**e**–**g**). Transverse sections of stems of the five-week-old control plant (**e**) and *StCLE8*- and *StCLE12*-overexpressing plants (**f**,**g**). Scale bar = 100 μm. ph—phloem cells; v—vessels; lp—lignified parenchyma; c—cambium cells. The vascular bundle in (**a**–**d**) is circled.

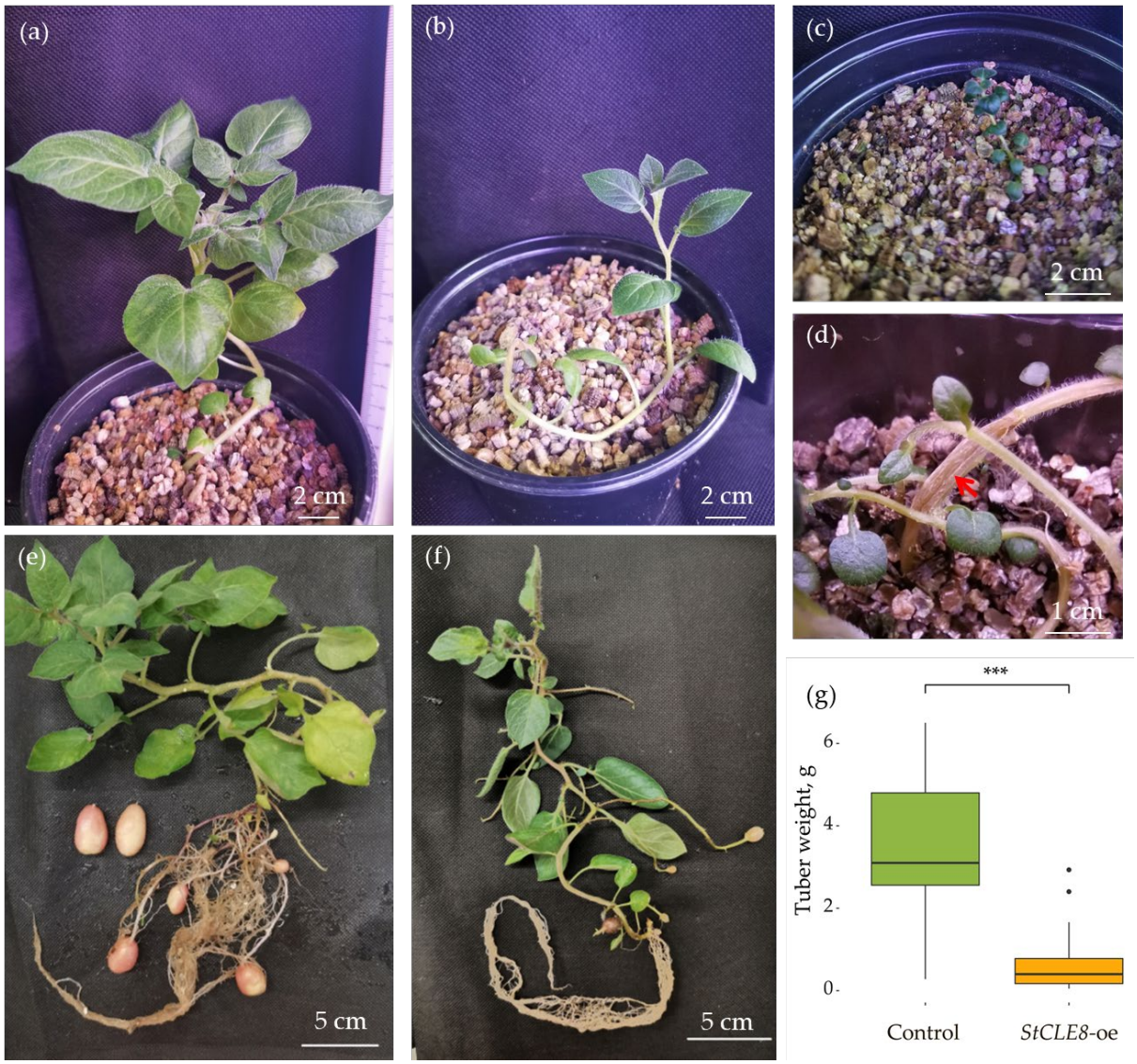

Figure 3. Phenotype of *StCLE8*-overexpressing plants. (**a**–**c**) Six-week-old control (**a**) and *StCLE8*-overexpressing (**b**,**c**) plants. (**d**) Stem damage (red arrow) due to excessive proliferation of cells in *StCLE8*-oe. (**e**,**f**) Two-month-old control (**e**) and *StCLE8*-overexpressing (**f**) plants. (**g**) Effect of *StCLE8* overexpression (*StCLE8*-oe) on tuber weight. ***, *p*-value < 0.001. Boxplots were generated using boxplot package in R.

At the same time, we observed that the *StCLE19*-oe potato plants were unable to produce adventitious roots (Figure S3). Normally, during *Agrobacterium*-mediated transformation, the induction of roots from transformed potato shoots is achieved in a hormone-free MS medium supplemented only with cefotaxime. Typically, root formation occurs within 5 days, with 91% of the shoots rooting successfully [24]. However, *StCLE19*-oe plants did not form any roots for several months. Additionally, structured cambium cell layers were not detected in their vascular bundles (Figure 2d).

3.3. Transcriptome Analysis of Transgenic Potato Stems with StCLE12 and StCLE19 Overexpression

In order to identify the potential targets of phloem-expressed potato *CLE*s, we performed a transcriptome analysis of *StCLE12*-oe and *StCLE19*-oe stems. We searched for differentially expressed genes (DEGs) using DESeq2 with padj < 0.05 and |log2 fold change| > 1 (Figure S4,

Table S2). Furthermore, we conducted a gene ontology (GO) analysis of the DEGs to investigate the role of *StCLE19* and *StCLE12* in plant development (Figure S5).

The transcriptome analysis revealed that a total of 1308 genes were upregulated, and 2474 genes were downregulated in the *StCLE19*-oe plants. Among the DEGs in the *StCLE19*-oe plants, there were 36 downregulated genes directly related to the formation of the plant's conductive system and 38 downregulated genes involved in root development.

For example, the central regulator of cambium activity, *StWOX4*, and two genes encoding TDIF receptors, *StTDR* and *StPXL*, were downregulated in *StCLE19*-oe plants. Additionally, among the downregulated genes, there were components of the WOX14-TMO6-LBD4 pathway that acted downstream of the TDR receptor, including the potato homologs, *WUSCHEL HOMEOBOX RELATED14* (*WOX14*) and *TARGET OF MONOPTEROS 6* (*TMO6*), and the target of the WOX14 and TMO6 transcription factors, *LATERAL ORGAN BOUNDARIES DOMAIN4* (*LBD4*) [25]. Interestingly, two homologs of *TMO6* and two homologs of *LBD4* were among the downregulated genes in *StCLE19*-oe plants. Additionally, four *Sieve Element Occlusion-Related* (*SEOR*) family genes, which encoded structural proteins in the sieve tubes, were also downregulated in *StCLE19*-oe plants [26], further implicating StCLE19 in the regulation of phloem element formation.

Several downregulated genes in *StCLE19*-oe plants were close to *Arabidopsis* genes, encoding regulators of auxin homeostasis, such as Tryptophan aminotransferase-related 2 (TAR2), an enzyme involved in IAA biosynthesis [27], Aux/IAA repressors of auxin-responsive gene transcription [28], and the AUX/LAX family of auxin influx transporters [29]. Additionally, this functional group included genes directly involved in the control of root development, such as DA1-RELATED 2 (DAR2)/lateral root development 3 (LRD3), a regulator of early phloem development and root system architecture [30], and SCARECROW (SCR), which establishes the root stem cell niche [31]. Interestingly, the expression of *StCLE6* was also downregulated in the stems of *StCLE19*-oe plants.

In the stems of *StCLE12*-oe plants, 975 genes were downregulated, and 888 were upregulated. Among the downregulated genes, there were 34 *SAUR*-like genes. The *small auxin up-regulated RNA* (*SAUR*) family is the largest family of early auxin response genes involved in various developmental processes [32]; thus, the decrease in *SAUR* expression could indicate the repression of auxin response. This is further supported by the downregulation of genes from the Aux/IAA family, which are also known as early-auxin-responsive genes [28].

Moreover, our analysis of the *StCLE12*-oe plant transcriptome revealed a significant number of genes that were associated with cell wall formation and rearrangement. This group includes genes encoding cell wall-associated enzymes, such as pectin lyases, pectate lyases, pectin methylesterases, expansins, and xyloglucan endotransglucosylase [33], as well as FASCICLIN-like arabinogalactan proteins, which are involved in secondary cell wall biogenesis [34]. The downregulation of these genes may contribute to the observed decrease in the number of lignified conductive elements in *StCLE12*-oe plants.

In addition, three *StPXC* genes encoding receptor-like kinases were found to be downregulated in *StCLE12*-oe potato plants. These PXY-correlated (PXCs) kinases were known to regulate secondary cell wall formation and vascular development through the TDIF-TDR/PXY signaling pathway, although their exact role is not fully understood [35,36].

Furthermore, several *CLE* genes (*StCLE17*, *StCLE16*, and *StCLE23*) were also downregulated in *StCLE12*-oe stems. While the function of StCLE23 has been previously studied and shown to be involved in the response to dehydration [37], the functions of StCLE17 and StCLE16 have not yet been investigated. Interestingly, these three genes were also downregulated in *StCLE19*-oe stems, suggesting a potential overlap in the function of phloem-specific StCLEs.

3.4. Promoter Activity of the StCLE8 and StCLE19 Genes in Potato

Next, we analyzed the promoter activities of *StCLE8* and *StCLE19*. According to the data from the phloem transcriptome analysis, both were found to be active in the phloem cells of potato tubers and stems (Figure 4).

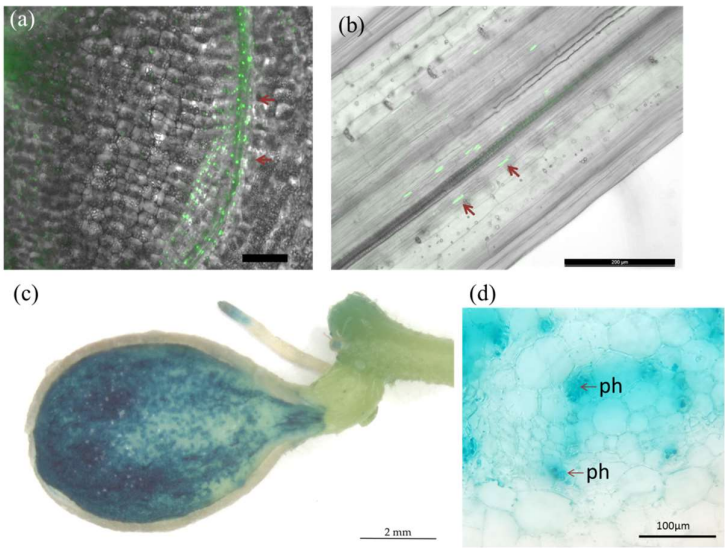

Figure 4. Localization of *StCLE8* and *StCLE19* expression in potato. (**a**,**b**) The *StCLE8* promoter activity (*promStCLE8::eGFP-NLP*) in the longitudinal section of the tuber (**a**) and stem (**b**). The red arrows indicate the presence of GFP in the nucleus of a phloem cell. (**c**,**d**) The expression of *promStCLE19::GUS* in the longitudinal section of the tuber (**c**) and cross-section of the stem (**d**) in potato. ph—phloem cells. Scale bar (**a**,**b**) = 100 µm.

During the cloning of the *StCLE19* gene promoter, we observed a difference in approximately 650 nucleotides between the promoter sequence in the Desiree potato cultivar and the reference genome in the database. This fragment (~650 bp) was found to be absent in tomato and other potato species (*S. commersonii*, *S. verrucosum*, *S. bukasovii*, *S. chaucha*, *S. juzepczukii*, *S. curtilobum*, and *S. ajanhuiri*), except for the reference species (*S. tuberosum* group Phureja DM1-3 516 R44) (Figure S6).

Furthermore, we compared the sequences of *StCLE19* genes in different potato species and observed that while the CLE19 domain sequence, corresponding to the mature StCLE19 peptide, was conserved across all species, there was polymorphism in sequences outside of this domain (Figure S7, [38]).

4. Discussion

The aim of this study was to investigate the role of phloem-expressed genes in potato development and growth, specifically in stem vascular bundles and tuber formation. We observed the high phloem-specific expression of the *StCLE12* and *StCLE19* genes. Further analysis revealed that StCLE12 and StCLE8 contained the same CLE domain as TDIF peptides, while the CLE domain of StCLE19 is similar to that of AtCLE25.

The overexpression of the *StCLE8*, *StCLE12*, and *StCLE19* genes in potato showed that all of them affected the development of the plant's conducting system. Genes encoding TDIF-like peptides caused an overproliferation of cambium cells that, in some cases, led to stem damage and plant death. Additionally, the overexpression of *StCLE8* caused a bushy phenotype, an increased number of tubers, and a delay in leaf aging. These effects are consistent with previous studies of TDIF effects in *Arabidopsis* [39–41], radish [42–44], poplar [45], and *Marchantia* [46]. However, the formation of aerial tubers, as a unique characteristic of potato, and stem damage as a result of excessive cambium proliferation were not previously observed.

In addition to these phenotypic effects, the overexpression of the TDIF-encoding *StCLE12* gene also resulted in changes in the expression levels of approximately 2000 genes. The most significantly downregulated genes were those involved in cell wall modification, such as expansins and pectin-lyases, as well as early auxin-responsive genes like *SAUR*s. Since the activity of cell wall enzymes is crucial for the differentiation of conductive elements [47], the downregulation of these genes may contribute to the known inhibitory effect of TDIFs on xylem differentiation [48,49]. At the same time, the downregulation of *SAUR* genes could indicate a decrease in the auxin response of *StCLE12*-oe plants. The evidence of a relationship between TDIF and auxin homeostasis was previously obtained in other plant species. For instance, in poplar, TDIFs, both IAA biosynthesis and sensitivity were enhanced to exogenous auxins [45], and in Arabidopsis, TDIF peptides specifically upregulated the transcript abundance of *PIN* genes that encoded auxin transporters [50]. Both effects are necessary for the TDIF-dependent stimulation of lateral and adventitious root formation [45,50]. However, our experiments on potato suggest the opposite effect of TDIF on the sensitivity of tissues to auxin.

The other phloem-expressed *StCLE* that we studied is closely related to *Arabidopsis* CLE25. We found that the overexpression of *StCLE19* inhibited adventitious root formation and also influenced cambium proliferation in the vascular bundles of potato stems. However, *StCLE19* gene overexpression had the opposite effect on root formation compared to the closely related *AtCLE25* and *AtCLE26* genes, which stimulated root growth [51]. The overexpression of *StCLE19* led to the downregulation of numerous genes involved in the regulation of cambium and phloem specification, as well as lateral root development. Therefore, despite their similarity in the CLE domain, StCLE19 was assumed to perform different functions compared to AtCLE25. Additionally, AtCLE25 transmits water-deficiency signals through vascular tissue in *Arabidopsis*, affects abscisic acid biosynthesis, and induces stomata closure (while AtCLE26 does not) [23]. These findings suggest that even a single amino acid change in the CLE domain can significantly impact the CLE function. Therefore, we hypothesized that the substitution of lysine with arginine at position 2 of the CLE domain, which distinguishes StCLE19 from AtCLE25, could contribute to its opposite function in phloem differentiation and lateral root development.

5. Conclusions

In summary, we analyzed the function of phloem-expressed potato *CLE* genes and identified a number of effects in their overexpression on plant-phenotype and gene-expression profiles. The data obtained were only partially consistent with data on relatively similar groups of CLEs from other plants, particularly for *Arabidopsis* CLEs. We hypothesize that this could be due to both sequence differences and the specificity of the object. This work contributes to the understanding of how CLE peptides function in various plants.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae9121265/s1. Figure S1: Six-week-old control and *StCLE12*-overexpressing plants; Figure S2: Overexpression of *StCLE8* resulted in a delay in leaf senescence; Figure S3: Effects of overexpression of the *GFP* and *StCLE19* genes on the root formation; Figure S4: The volcano plots of DEGs in the *StCLE12*-oe and *StCLE19*-oe plants; Figure S5: Most significantly enriched GO pathways in genes upregulated and downregulated in the *StCLE12*-oe or *StCLE19*-oe stems; Figure S6: Alignment of the *CLE19* promoter region sequences; Figure S7: Alignment of the CLE19 pre-propeptide sequences; Table S1: List of primers; Table S2: Transcripts per kilobase million (TPM) values of genes in phloem in the potato stem. Differentially expressed genes (DEGs) in the stems of *StCLE19*- and *StCLE12*-overexpressing plants.

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Data Availability Statement: Sequence reads of the RNA-Seq data generated in this study are available in the National Center for Biotechnology Information database, under the BioProject accession number PRJNA1030186.

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

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