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Conserved mechanism for perception of root-active CLE peptides

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19   **Abstract**

20   Precision in sensing the environmental cues and adjusting the growth and the physiology of the root

21   system is necessary for plant robustness. Plants achieve their phenotypic plasticity by tightly

22   controlling and buffering developmental decisions. In addition to the classical plant hormones, the

23   CLE peptides are exceptionally important in mediating development, and responses to environmental

24   stresses. While the CLV3-CLV1 module appears to be highly conserved to control the proliferation of

25   the shoot apical meristem stem cells, we do not know whether the function of root-specific CLEs that

26   are implicated in vascular development, in mediating drought stress, sugar starvation, phosphate and

27   nitrate deficit in Arabidopsis is also conserved in other plant species. Here we present a careful re-

28   analysis of the CLE signaling components in the tomato genome and show that the mechanism of

29   root-active CLE peptides is deeply conserved in Arabidopsis and tomato. Due to the small gene size

30   and high sequence variability, it is extremely difficult to precisely annotate *SlCLE* genes in plant

31   genomes. Our analysis of the *SlCLE* family, based on a combination of iterative tBLASTn and

32   Hidden-Markov-Model (HMM), revealed thirty-seven new *SlCLE*s in addition to the fifteen reported

33   previously. Analyzing publicly available RNAseq datasets, we found that the majority of *SlCLE* genes

34   are preferentially expressed in root tissues. We could confirm the biological activities of selected

35   SlCLE peptides which had a conserved potency like their Arabidopsis orthologs to suppress primary

36   root growth. We show, that root responses are mediated by *SlCLAVATA2*, indicating the conservation

37   of CLE perception mechanism.

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39   **One-sentence summary**

40   Using a combination of iterative tBLASTn and Hidden-Markov-Model approaches, we uncovered 37

41   new tomato *CLE* genes predominantly expressed in roots, and we showed a conserved effect on root

42   meristem arrest, that was *SlCLAVATA2*-dependent.

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Highlights

- We applied a combined approach of iterative tBLASTn and Hidden-Markov-Model to identify fifty-two tomato *SlCLE* genes, including thirty-seven new genes

- All identified genes encode for pre-propeptides with a single CLE-domain containing conserved residues similar to Arabidopsis

- Analyzing the publicly available RNAseq datasets, we could confirm the expression of *SlCLE* genes that was often associated specifically with root or shoot, a certain developmental stage of the fruit, or with drought stress conditions

- Remarkably, the majority of *SlCLE* genes are predominantly expressed in the root tissues

- We showed the conserved inhibitory effect on the root meristem and columella cells division for the selected SlCLE peptides that were *SlCLAVATA2*-dependent.

57 **1.   Introduction**

58   Plant roots explore the soil for water and minerals that are distributed irregularly in space and time.

59   They grow towards the resources and limit the growth towards the unfavorable substances [1].

60   Remarkably, roots integrate the inputs from the soil environment, to adjust the growth and to

61   maximize the nutrient and water uptake. The biology of the root growth and responses to the

62   environment is an emerging topic [2] and therefore, finding new regulators and pathways that underlie

63   the root growth under stressful conditions is extremely important. In the last decades, the Arabidopsis

64   research yielded many key players that shape the root system, including plant hormones, small RNAs,

65   transcription factors, and small signaling peptides [3, 4]. At the same time, we still do not know

66   whether the mechanisms observed in Arabidopsis are fully conserved in the eudicot and the monocot

67   crop species.

68   One of the most studied groups of hormone-like peptides that have been shown to mediate root

69   vascular development, root stem cell niche maintenance, and responses to the availability of water,

70   phosphate, nitrate in the soil, sugar levels, and root-to-shoot communication is the

71   CLAVATA3/EMBRYO-SURROUNDING REGION-RELATED (CLE) family [5-12]. The CLE

72   genes are relatively small and encode for non-functional pre-propeptides of about 100 amino acids.

73   To become active peptides, additional processing, including cleavage by subtilases [13], and, often

74   prolines hydroxylation and glycosylation are necessary[13-16]. CLE peptides are secreted to the

75   apoplast, where they are perceived by the Class XI of the leucine-rich repeats receptor-like kinases

76   (LRR-RLKs) [9, 17]. In Arabidopsis, in addition to the CLAVATA1 receptor, three BARELY ANY

77   MERISTEM (BAM) receptors have been shown to bind CLE peptides. These receptors have three

78   domains: an extracellular domain, which is responsible for the binding of the ligand, a transmembrane

79   domain, which anchors the receptor in the plasma membrane, and a cytoplasmic kinase domain, that

80   triggers the intracellular signaling by phosphorylating downstream targets. Moreover, the receptor-

81   like kinases CLV3 INSENSITIVE KINASES (CIKs) act as co-receptors both in perceiving the root-

82   active CLE peptides and in the CLV3 signaling in the shoot apical meristem [18, 19]. In addition to

83   these cognate receptors, it has been shown, that also LRR receptor-like protein (LRR-RLP) named

84   CLAVATA2 (CLV2) creates a dimer with the pseudo-kinase CORYNE (CRN) to perceive the full

85   range of CLE peptides in the Arabidopsis root [9].

86   The unique roles of CLE peptides in mediating shoot and root growth and adaptation to

87   environmental stresses underlies the special interest of the scientific community to study this group of

88   ancient and highly conserved plant peptide hormones not only in model plants but also in crop

89   species. The genome-wide analyses of *CLE* genes have been performed in many plant genomes,

90   including tomato, rice, wheat, maize, soybean, grape, potato and cucumber [20-23]. Due to the small

91   gene size and high sequence variability, the annotation is challenging. It has been previously

92   reported, that in the tomato genome there are only 15 CLE genes [23]. Among them, *SlCLV3* and

93   *SlCLE9* encode for signaling peptides that need to be arabinosylated to control the stem cell

94   proliferation and shoot apical meristem size. Remarkably, the tomato domestication mutation

95   *fascinated (fas)* that led to the increased fruit size, is a result of disruption of the *SlCLV3* promoter

96   that led to the reduction in the gene expression [24]. The *SlCLE9* is the closest paralog of *SlCLV3* and

97   can actively compensate for the absence of *SlCLV3* to buffer the impact on the stem cell niche [24,

98   25]. The unraveling of additional tomato *CLE* genes and more careful phylogenetic analysis is

99   necessary to fully understand the role of these conserved ligands in tomato development and

100   adaptation to the changing environment.

101   **2.   Materials and method**s

102   2.1 *SlCLE*s identification

103   2.1.1 Iterative tBLASTn

104   All previously described *Arabidopsis thaliana* CLE full-length protein (pre-propeptide) sequences

105   were used as queries to search by tBLASTn in *Solanum lycopersicum* genome SL3.0 and SL4.0 in the

106   plant section of the EnsemblGenome database [26]. The hits were then used to search by BLASTp in

107   closely related species of the Solanaceae family (*Nicotiana attenuata*, *Solanum tuberosum,* and

108   *Capsicum annuum*). The newly identified CLE proteins were exploited to identify by tBLASTn

109   additional similar sequences in tomato's genome, which were then used to search again in the above

110   Solanaceae-species genomes. Between each iteration, candidate loci were individually confirmed

111   based on the CLE domain sequence and the presence of a signal peptide sequence in 5'.

112   2.1.2 Hidden-Markov-Model approach

113   A list of 256 CLE proteins obtained in multiple species (*A. thaliana, N. attenuata*, *S. tuberosum*,

114   *Populus trichocarpa, Medicago truncatula, Brachypodium distachyon,* and tomato sequences found

115   in 2.1.1) was aligned with MEGA X [27] and used to build an HMM with HMMER3 [28]. The

116   HMM was used to search *S. lycopersicum* SL4.0 genome with Genewise [29] (the genome was split

117   in chunks of 9 million bp with EMBOSS splitter & seqretsplit [30]). This led to a list of 61 CLE

118   candidates that was concatenated with the 40 CLE of 2.1.1 above. After manual cleaning and

119   removing duplicates, we confirmed a clean list of 57 *CLE* candidates.

120   2.1.3 Candidate verification

121   The gene structure of the 57 candidate *CLE* was verified by tBLASTn and BLAT [31] against the

122   SL3.0 genome as in 2.1.1 and by manual evaluation of the resulting hits for the correctness of their

123   exon-intron structure. Five pseudogenes could be identified (with in-frame stop codons or no initiator

124   methionine), leaving a final list of 52 *CLE* genes.

2.2 Transcriptomic analysis

We selected four publicly available RNAseq and TRAPseq datasets to search for expression clues of the CLE genes in various tissue types of *S.lycopersicum* M82: RNAseqA [32], RNAseqD [33], RNAseqF1 and RNAseqF2 [34], TRAPseq [35].

The selected samples of all the four datasets were remapped to the SL4.0 genome assembly with bwa [36] and samtools [37] to obtain sorted bam files. A Bed file containing the CLE gene positions was created (**CLEgene.bed**) and used to count the reads per gene with bedtools multicov [38]. A heatmap of the logTPM (transcripts per million) for CLE genes counts over all genes was created with a custom-made R script (**script**) for each dataset.

2.3 Phylogenetic analysis

Alignments of the CLE proteins sequences were performed in MEGA X [27], using ClustalW (Figure 2) or MUSCLE (Figure S1-S2), and manually corrected. The phylogenetic trees were generated by IQTREE with 1000 bootstrap replicates [39], and visualized with iTOL [40].

2.4 Plant material and treatments

2.4.1 Mutants and seed sterilization

Seeds of *Solanum lycopersicum* M82 were surface-sterilized with a sterilization solution (2.5% sodium hypochloride, 0.1% Tween-20) for 20 minutes. Seeds of *Arabidopsis thaliana* Col-0 were surface-sterilized with 70% ethanol and 0.05% Triton-X100 solution for 3 minutes. Immediately after, the seeds were washed with sterile distilled water five times. Tomato (*Slclv1*-a2, *Slbam1*-a1, *Slbam4*-a2 and *Slclv2-5*) and Arabidopsis (*Atcrn-10*) mutants are CRISPR-mediated mutants previously described [25, 41].

2.4.2 Root assays

S. lycopersicum sterilized seeds were placed on 24 cm square plates containing 1μM of the indicated SlCLE peptide. After 2 days in the dark, plates were placed vertically in 16h light / 26 ˚C – 8h dark / 24 ˚C cycles for a week. *A. thaliana* sterilized seeds were grown onto 12cm square plates containing 50nM of indicated AtCLE peptides. After 2 days in the dark at 4 ˚C, plates were placed vertically in 16h light– 8h dark cycles at 22 ˚C for a week. The plates were scanned at high resolution, and primary root length was measured with the "simple neurite tracer" tool on Fiji (www.imagej.net). All CLE peptides are synthetic un-modified peptides at >75% purity (www.genscript.com) solubilized in water at 10mM stock concentration.

2.4.3 Tomato drought stress assay

156   The assay was modified from a published protocol of hydroponically grown tomato [42]. In brief,
157   sterilized tomato seeds were placed on moistened blotting paper and kept in dark at 26˚C for 3 days.
158   Germinated seeds were placed on Eppendorf-type tubes with cut end filled with 0.6% water-agar in
159   16h light / 26 ˚C – 8h dark / 24 ˚C cycles and high humidity environment for one week. Then, the
160   seedlings were transferred to hydroponics containers, in which the roots grow in an oxygenated
161   Hoagland solution in darkness. The nutritive solution was renewed every week. After 3 weeks, one
162   day after replacing the nutrient solution, drought stress was induced with a fresh solution
163   supplemented with 15% PEG-6000. Three different containers were used for the experiments
164   generating each 2 biological replicates. Each biological replicate is a pool of 2 to 3 plants from the
165   same container. The root samples contain all the root system coming out of the Eppendorf. The shoot
166   samples contain all the leaves and around 5 cm of stem harboring the shoot apical meristem, thus
167   these samples do not contain the main stem which has been strongly lignified.

168   2.5 Quantitative RT-PCR of tomato CLE genes

169   Plant tissues were rapidly shock frozen in liquid nitrogen. Frozen samples were grinded using mortar
170   and pestle. Total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma). The remaining
171   DNA was eliminated by DNAse I treatment (Jena-Bioscience) and with a 2M LiCl precipitation. The
172   absence of the genomic DNA in the RNA samples was tested by PCR. cDNA synthesis was
173   performed using the SensiFAST cDNA synthesis kit (meridian). Quantitative PCRs were performed
174   using Fast Start Universal SYBR-green Master (Roche), with primers indicated in Supplemental
175   Table 1. The thermal cycler (Mic qPCR Cycler, biomolecular systems) conditions were: 95˚C 2 min,
176   45 cycles of 95˚C 15s, 58 ˚C 10s, 60 ˚C 50s, followed by a dissociation curve analysis. The
177   expression level was normalized to Actin on 6 biological replicates.

178   2.6 Microscopy

179   About 1 cm of the primary root tips of one-week-old tomato seedlings were fixed with 4%
180   paraformaldehyde in a 1xPBS solution for a minimum of 6 hours. After 2 washes in 1xPBS, the
181   samples were cleared in a ClearSee solution [43] for one week. Subsequently, to visualize the cell
182   walls, the calcofluor white staining was performed with 0,02% calcofluor-white dissolved in the
183   ClearSee solution for 2 days, followed by two washing steps with ClearSee. Samples were incubated
184   in ClearSee solution for a minimum of 2 weeks before imaging. Images were taken with a confocal
185   laser scanning microscope (Leica SP5). The calcofluor-white stained cell walls were excited at 405
186   nm and emitted light detected at 415-500nm. These images were used to quantify root width in the
187   differentiation zone, columella length and cell number in Fiji (www.imagej.net).

188   2. 7 Statistics

189   Statistical analysis was performed using Rstudio (www.rstudio.com) after log transformation of the

190   data. Statistical significance was analyzed by ANOVA, and followed by a post-hoc Tukey test to

191   determine the different statistical groups.

192   **3.   Results**

193   **3.1 Identification of 37 new *SlCLE* genes**

194   Motivated by uncovering the role of CLE peptides in tomato roots, we performed a re-analysis of the

195   CLE peptide-encoding genes. The first genome-wide analysis in the tomato genome revealed only

196   fifteen *SlCLE* genes [23] and further attempts failed to uncover additional genes [21, 22]. In our

197   study, we applied a combined bioinformatic approach to identify additional *SlCLE* genes using the

198   most recent versions of the tomato reference genome SL3.0 and SL4.0 [44]. Firstly, we performed an

199   iterative tBLASTn search on the full genome which revealed forty *CLE* genes (Figure 1B). Secondly,

200   we applied a Hidden-Markov-Model, that resulted in forty-seven *CLE* genes. Remarkably, for a total

201   of 52 *CLE* genes in tomato, fifteen were uncovered by only one of the two approaches. This indicates

202   that the combination of these two methods can be instrumental to find short genes like *CLEs* in plant

203   genomes.

204   Tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) belong to the same family and the

205   *CLE* genes in both share high gene sequence similarities. To further estimate how successful our

206   approach was to find *CLE* genes in tomato, we compared their sequence homologies with the 41

207   recently annotated members in potato [20] by phylogenetic analysis (Supplemental Figure 1). Except

208   for *StCLE2* and *StCLE5*, we found orthologous for all the other CLE genes in the genome of potato.

209   This result indicates that our analysis uncovered most of the *CLE* genes in the tomato genome. In the

210   same study [20], the authors report about additional 16 new CLE genes in tomato, which confirms

211   some of the new genes uncovered in our comprehensive study.

212   We mapped the *SlCLE* genes on tomato's chromosomes (Figure 1D). We numbered the identified

213   *SlCLE* genes as follows: the previously reported fifteen genes are numbered *SlCLE1-SlCLE15*. The

214   newly identified genes (*SlCLE16* to *SlCLE52*) are numbered according to their chromosomal location,

215   starting from chromosome 1 (Figure 1D). *SlCLE* are diversely present on all 12 chromosomes in

216   tomato, from a single gene on chromosome 6 to up to nine genes on chromosome 5. The fact that

217   several *SlCLE* genes are located in high proximity with each other's, forming gene clusters, and

218   showing high sequence similarity, suggest that they arise from tandem gene duplication events. To

219   investigate the gene structure diversity of tomato *CLE*s, the exon-intron composition was predicted

220   based on sequence homologies (Figure 1A). In addition, we used publicly available RNAseq datasets

221   [32-34, 45, 46], from root, shoot and fruit samples, to support these gene structure predictions. Reads

222   were mapped on the anticipated coding region of 28 *CLE* genes out of the 37 newly uncovered loci

223   (Figure 1A). Overall, the tomato *SlCLE*s have a single CLE domain in the 3' of the coding region and

do not include any intron (Figure 1A). In the case of *SlCLE31*, an insertion of a single nucleotide in the tomato genome SL4.0, which is not present in the version SL3.0, creates a frameshift in the CDS suggesting that it is a pseudogene. However, Sanger sequencing of this particular locus confirmed the correctness of the sequence in the SL3.0 genome. Furthermore, to evaluate to what extend the CLE motif is conserved between Arabidopsis and tomato, we created sequence logos (Figure 1C). The CLE domain is extremely well conserved, including the prolines at positions 4, 6, and 9, as well as the arginine at position 1, glycine at positions 6, and histidine-asparagine/histidine at positions 11-12.

In parallel, we searched for the CLE receptor genes, namely *CLV1*, *BAM1*, *BAM2*, *BAM3,* and *PXY*. As previously reported in tomato [25], we found one copy of *SlCLV1*, four *SlBAM* homologs, two *SlPXY*-like genes, one *SlPXL1,* and one *SlPXL2* (Supplemental Figure 2).

3.2. Expression analysis of *SlCLE*s and their diversification

Next, we wanted to confirm, that the identified genes are expressed in tomato based on the publicly available RNAseq datasets [32-34, 45, 46]. Remarkably, the majority of *SlCLEs* show predominant expression in root tissues, while some are shoot-specific or evenly expressed in both (Figure 3A). We could observe, that *SlCLE20*, *SlCLE2, SlCLE40* and *SlCLE41*, for example, are root-specific genes and their expression increases with the age of the plant. Using qPCR, we could detect that *SlCLE5*, *SlCLE21*, *SlCLE40* have higher expression in the root tissues, while *SlCLE13*, *SlCLE32*, *SlCLE45,* and *SlCLE52* are more expressed in the shoot tissues (Figure 3B).

To explore the diversification of the tomato *CLE* genes, we created a phylogenetic tree of the full-length proteins from tomato and Arabidopsis (Figure 2). This analysis revealed gene sub-groups that are conserved in both plant species as well as showed unique genes, which could pinpoint *CLE* diversifications in tomato or losses in Arabidopsis, and define orthology. Interestingly, in tomato, we found nine homologs of Arabidopsis CLE8 (Figure 2). In Arabidopsis, this peptide is expressed and acts specifically during embryo and endosperm development [47], but the roles of the nine orthologs in tomato are yet to be uncovered. For phloem CLE peptides in Arabidopsis, which includes CLE25, CLE26, CLE45 we found seven orthologs in tomato, which also suggests the diversification of the phloem genes.

3.3 The expression of *SlCLEs* in the developing fruit and in water deficit stress

To test whether *SlCLE* genes are expressed during fruit development, we analyzed the dataset from [33]. In this study, wild type M82 and yellow-fruited *yft1* mutant fruits were sampled at different developmental time points, from 35 to 60 days-post-antherisation. Our analysis shows, that *SlCLE12*, *SlCLE30*, *SlCLE31*, *SlCLE34*, and *SlCLE38* are the most expressed in tomato fruits independently of the genotype, whereas *SlCLE5, SlCLE11, SLCLE51* expression is impaired in the yellow-fruited *yft1*

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257   mutant (Supplemental Figure 3A). These results suggest, that *SlCLE* genes could play a role during

258   tomato fruit ripening.

259   Numerous studies showed that CLE peptides mediate abiotic stress signals, for example, CLE25

260   peptide in Arabidopsis was shown to be induced during dehydration, moving from root to shoot as a

261   mobile signal, triggering ABA biosynthesis and stomatal closure [5]. Therefore, we wanted to test,

262   whether some *SlCLE* genes are up-regulated under drought stress conditions. First, we analyzed the

263   dataset published previously and could find several genes that are specifically expressed under

264   drought in the tomato leaves (Supplemental Figure 3B) [32]. *SlCLE1*, *SlCLE12*, *SlCLE32, SlCLE45*

265   and *SlCLE52* showed an increased expression (Supplemental Figure 3B), suggesting that they could

266   be involved in adaptive responses to water deficit We wanted to test, whether these genes are quickly

267   up-regulated, also following short osmotic stress. To this end, hydroponically grown tomato plants

268   were treated with a 15% PEG6000 solution for one hour, and roots and shoots samples were collected

269   separately. Since Dehydrins (DHN) play a key role in plant response and adaptation to water deficit

270   conditions and are accumulated during drought stress, we used the *SlDehydrin (SlDHN)*

271   (*Solyc02g084850*) expression as a control to monitor the effect of water deficit in our experiment.

272   After one hour, *SlDHN* was strongly upregulated both in root and shoot tissues of treated tomato

273   plants (Supplemental Figure 3C). However, we could not detect a significant induction for those

274   *SlCLE* genes (Supplemental Figure 3C). Next, we wanted to test, whether similarly to Arabidopsis,

275   the tomato orthologs of *AtCLE25* are upregulated in roots to mediate a dehydration response like it

276   has been demonstrated in Takahashi et al 2018 [5]. We could not detect any significant induction in

277   *AtCLE25* orthologs in tomato under this short osmotic stress (Supplemental Figure 3D). One

278   possibility is that our experimental settings were not enough to trigger similar osmotic stress like

279   reported in [5] and [32]. Another possibility is that in tomato, none of the *AtCLE25* orthologs are

280   involved in mediating drought responses.

281   **3.4 A conserved effect of *SlCLE* peptides on root apical meristem.**

282   To test whether the predicted CLE peptides are biologically active, we performed root growth assays

283   with commercially synthesized unmodified peptides. It has been shown, that in Arabidopsis 20 out of

284   32 peptides affect the primary root growth, leading to root meristem arrest [9, 48]. To compare the

285   activity of orthologous CLE peptides, we tested their capacity to inhibit root growth. For this purpose,

286   we selected CLE peptides from different subgroups and well-supported orthologous genes in tomato

287   and Arabidopsis, which were known to have different potency in arresting the root meristem in

288   Arabidopsis. For example, the treatment with AtCLV3, AtCLE25, and AtCLE45 peptides at 50nM

289   triggers a strong reduction of the primary root length (Figure 4A right side), and this response

290   depends on the pseudo-kinase CORYNE and the receptor-like protein CLAVATA2 [9]. However, the

291   AtCLE9/10 and AtCLE22 peptides had a much smaller root growth inhibition effect in the wild-type.

292 Because tomato roots are much thicker, with a diameter of about 320 μm at the tip instead of

293 approximately 120 μm in Arabidopsis, tomato is less sensitive to external application of CLE

294 peptides. Therefore, we applied SlCLE peptides at a concentration of 1 micromolar. We could

295 observe, that in tomato roots, SlCLE15, SlCLE6/19 and SlCLE24 peptides (orthologous of AtCLV3,

296 AtCLE25, and AtCLE45, respectively) led to a strong reduction of the primary root growth (Figure

297 4A left side). In contrast, SlCLE30/31 and SlCLE45 peptides (orthologous of AtCLE9/10 and

298 AtCLE22, respectively) treatment did not trigger a significant reduction of the primary root length.

299 This result indicates that the amino-acid composition of CLE peptides is important for their biological

300 activity in the root; but, more importantly, that there is a conservation of the biological activity of

301 these CLE peptides between Arabidopsis and tomato, two evolutionary separated species.

302 To have more insight into the effect of SlCLEs on tomato roots, we analyzed the morphology of the

303 root tips (Figure 4B). We observed in three treatments (SlCLE15, SlCLE19, SlCLE24) not only the

304 decreased root length but also the reduction in the root diameter and the columella length. We also

305 looked at the number of columella layers and the columella cell length to understand whether the

306 treatment affects cell division or cell elongation. Root-active SlCLE peptides treatment led to a

307 reduction of columella layers, but not their average cell length, suggesting that cell division is

308 primarily affected (Figure 4C-F). We can conclude, that CLE peptides have a conserved role in

309 Arabidopsis and tomato roots by restricting the meristematic cell divisions. Next, we asked whether

310 this conserved inhibitory effect on the root is mediated by orthologous receptor-like kinases. It has

311 been shown in Arabidopsis, that such inhibitory root effect of CLE peptides is CLAVATA1-

312 independent, but CLAVATA2-dependent [48]. We have tested the previously published tomato

313 CRISPR mutants *clv1 bam1 bam4* and *clv2* [25] for their root sensitivity to SlCLE peptides. The

314 mutant *clv1 bam1 bam4* showed a strong sensitivity to SlCLE24 peptide (Supplemental Figure 4).

315 However, *clv2* seedlings were absolutely blind to the high concentrations of the peptide in the media.

316 This result reinforces the claim, that CLE peptides have a conserved activity across plant families and

317 that the perception mechanism is also conserved.

318 **4.   Discussion**

319 Signaling mediated by CLE peptides is highly conserved in the land plants. The precise control of the

320 shoot apical meristem stem cell niche by CLV3-CLV1 module is the most ancient pathway, whereas

321 additional CLE genes and additional receptor complex components evolved later, with establishing

322 vascular plants [49]. It seems, that the possible ancestral function of CLV3-like peptides was to

323 suppress the proliferation of the shoot apical meristem in early land plants (bryophytes). CRN and

324 CLV2 create a complex to mediate CLE signals, and additional CLEs appeared later in the vascular

325 plants. To understand the function of these "later" CLE genes in tomato, we performed a re-analysis

326 of these genes. We have discovered thirty-seven new *SlCLE* genes that have not been found in the
327 previous analyses and we could show that they are expressed and likely result in active peptides.

328 Strikingly, the perception of SlCLEs in the tomato root meristem is highly conserved and requires
329 SlCLV2 receptor-like protein. The loss-of-function *Slclv2* mutant is fully insensitive to the peptide
330 treatment. In tomato wild type roots, the root-active synthetic peptides (orthologs of Arabidopsis
331 CLV3, CLE25, and CLE45) inhibited the cell division rate in the columella and vascular tissues. This
332 result, again, indicates the conserved effect of SlCLEs on the root meristem.

333 It has been shown, that SlCLV3 and SlCLE9 undergo arabynosylation and are active at 60 nM
334 concentration while glycosylated and are not active at this concentration if the peptide is unmodified
335 [24]. Moreover, it has been demonstrated that the biological activity of Arabidopsis CLV3 gradually
336 increases in mono-, di- and triarabinosylated CLV3 glycopeptides, becoming equally active with non-
337 modified peptide at 1 μM concentration [50]. The synthesizing of the complex arabinose chain is
338 technically difficult and only a few laboratories in the world established such synthesis [50]. It is
339 plausible, that the effect of glycosylated SlCLEs on the root meristem will be visible at a much lower
340 concentration.

341 Similar to what has been shown in Arabidopsis, we showed that the root sensitivity to SlCLE
342 treatment was not dependent on *SlBAM1*, or *SlCLV1*. This result suggests that in tomato roots
343 SlBAM1 can act redundantly with additional SlBAMs, for example, with the closest homolog
344 *SlBAM2* and *SlBAM3* in the perception of SlCLE ligands. *Slbam4* loss-of-function mutant also
345 remains sensitive to the peptide treatment. This receptor-like kinase seems to be lost in the
346 Arabidopsis genome during the evolution and the function of this gene in tomato and other plants
347 species needs to be unraveled.

348 The phylogenetic analysis and the clustering of the *SlCLE* genes with the Arabidopsis orthologs
349 allowed us to detect very interesting diversification events. For example, we found nine orthologs of
350 *AtCLE8* that was shown to control embryo development [47]. The role of these genes remains to be
351 uncovered. In conclusion, our work draws a more precise picture of the components of CLE signaling
352 in this fleshy fruit crop plant paving a path for new discoveries.

363 **Competing interests**

364 The authors declare no competing interests.

365 **Figure legends**

366 **Figure 1. *CLE* genes identified in the tomato genome. A.** The gene structure of the tomato *CLE*

367 genes. Gene structures in dashed lines indicate the *SlCLE* genes for which no proof of expression was

368 found in root, shoot and fruit tissues. **B.** Venn-diagramm showing the number of *CLE* genes identified

369 by each method. **C.** Sequence logo of the conserved CLE domain in tomato and Arabidopsis using

370 WebLogo (https://weblogo.berkeley.edu/logo.cgi) (reference). The height of the bars represents the

371 conservation value of each amino acid at the given position. **D.** The chromosomal location of the

372 tomato *CLE* genes.

373 **Figure 2. The phylogenetic tree of the full-length CLE proteins from tomato and Arabidopsis.**

374 The groups of proteins sharing high similarity (clusters) are highlighted by background colors. The

375 names in red indicate the new *CLE* genes uncovered in this study. Nodes supported by bootstrap

376 values superior to 50 are indicated by dots of size proportional to the bootstrap values.

377 **Figure 3. Expression analysis of *SlCLE* genes in root and shoot tissues[34]. A.** Heatmaps of

378 log(TPM) of tomato *CLE* genes in the root (left) and the shoot (right) at 6, 9, and 12 days after

379 plantation from tomato grown in pots. **B.** Expression of selected *SlCLE*s in root and shoot tissues by

380 qPCR from 3 weeks old tomato plant grown in hydroponic conditions.

381 **Figure 4. Functional conservation of root active CLE peptides in tomato and Arabidopsis. A.**

382 Effect of Arabidopsis and tomato orthologous CLE peptides on the primary root length (PRL). **B.**

383 Representative confocal images of tomato primary root tips grown on mock or indicated SlCLE

384 peptide containing medium. The cell walls are stained with calcofluor white. Red and green arrows

385 indicate root width columella length, respectively **C-F.** Quantification of the indicated root tip

386 morphology characteristics from images show in B. Letters indicate different statistical groups

387 (ANOVA, followed by post-hoc Tukey test). **G.** Primary root length of wild type and *Slclv2* mutant

388 grown in presence of SlCLE6/19p and SlCLE24p. **H.** Representative confocal images of wild type

389 and *Slclv2* primary root tips grown on mock or indicated SlCLE peptide containing medium. The

390 scale bars in **B** and **H** correspond to 100μm.

391 **Supplemental Figures and Tables**

Supplemental Figure 1. Sequence homology between tomato and potato CLE proteins. Phylogenetic tree of full-length CLE proteins from tomato (red), potato (blue), *Arabidopsis thaliana*, *Medicago truncatula*, *Brachypodium distachyon*. Nodes supported by bootstrap values superior to 50 are indicated by dots of size proportional to the bootstrap values.

Supplemental Figure 2. Phylogenetic analysis of CLE receptors. The receptor genes from *Arabidopsis thaliana* and tomato are in bold black and red, respectively.

Supplemental Figure 3. Expression of tomato *CLE* genes in the developing fruit and following drought stress. A. Heatmaps of log (TPM) of *SlCLE* genes in the developing fruit at 35, 47, 54, and 60 days post antherisation (DPA), in wilt-type and *yellow fruit tomato1* mutant. Genes in red indicate *SlCLE* which are upregulated during fruit maturation in a *yft1* dependent fashion. **B.**Heatmaps of log (TPM) of *SlCLE* genes in plant leaves (M82 or a drought tolerant introgression line IL9-1) after a 10 days drought stress in pots. Genes in red indicate *SlCLE* which are upregulated by the drought treatment. **C.** Expression analysis by qPCR of selected *SlCLE* genes on root and shoot samples from 3 weeks old plants grown in hydroponic after 1 hour 15% PEG6000 treatment. **D.** Expression analysis of the orthologs of *AtCLE25* in response to osmotic stress induced by the PEG treatment.

Supplemental Figure 4. Effect of SlCLE24 peptide on the primary root length (PRL) in wild type (M82) and *clv1bam1bam4* mutant.

Supplemental Table 1. Primers used in this study.

Supplemental Table 2. Sequences and chromosome locations of the *SlCLE*s

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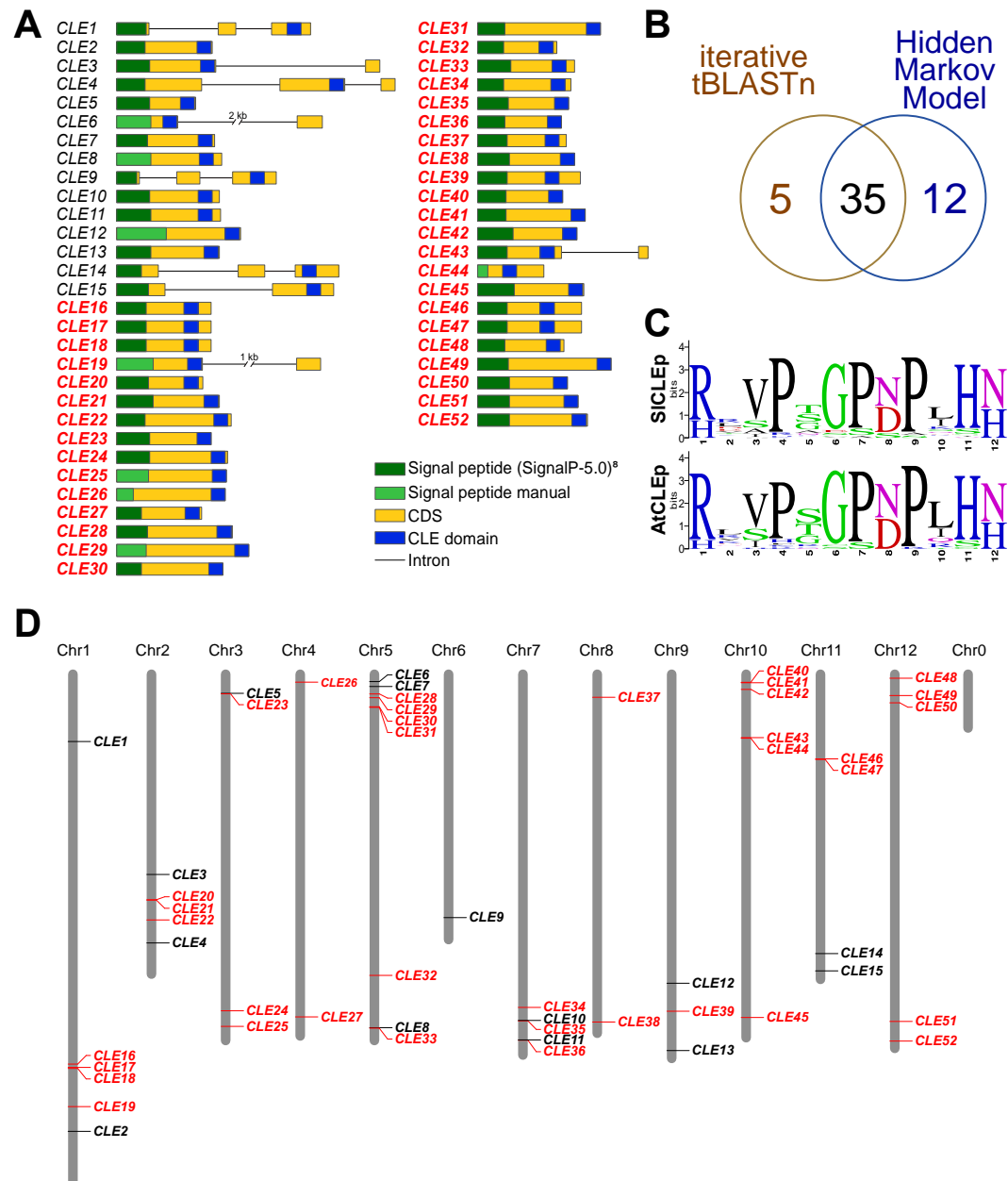518

Figure 1. *CLE* genes identified in the tomato genome. A. The gene structure of the tomato *CLE* genes. Gene structures in dashed lines indicate the *SlCLE* genes for which no proof of expression was found in root, shoot and fruit tissues. **B.** Venn-diagramm showing the number of *CLE* genes identified by each method. **C.** Sequence logo of the conserved CLE domain in tomato and Arabidopsis using WebLogo (https://weblogo.berkeley.edu/logo.cgi) (reference). The height of the bars represents the conservation value of each amino acid at the given position. **D.** The chromosomal location of the tomato *CLE* genes.
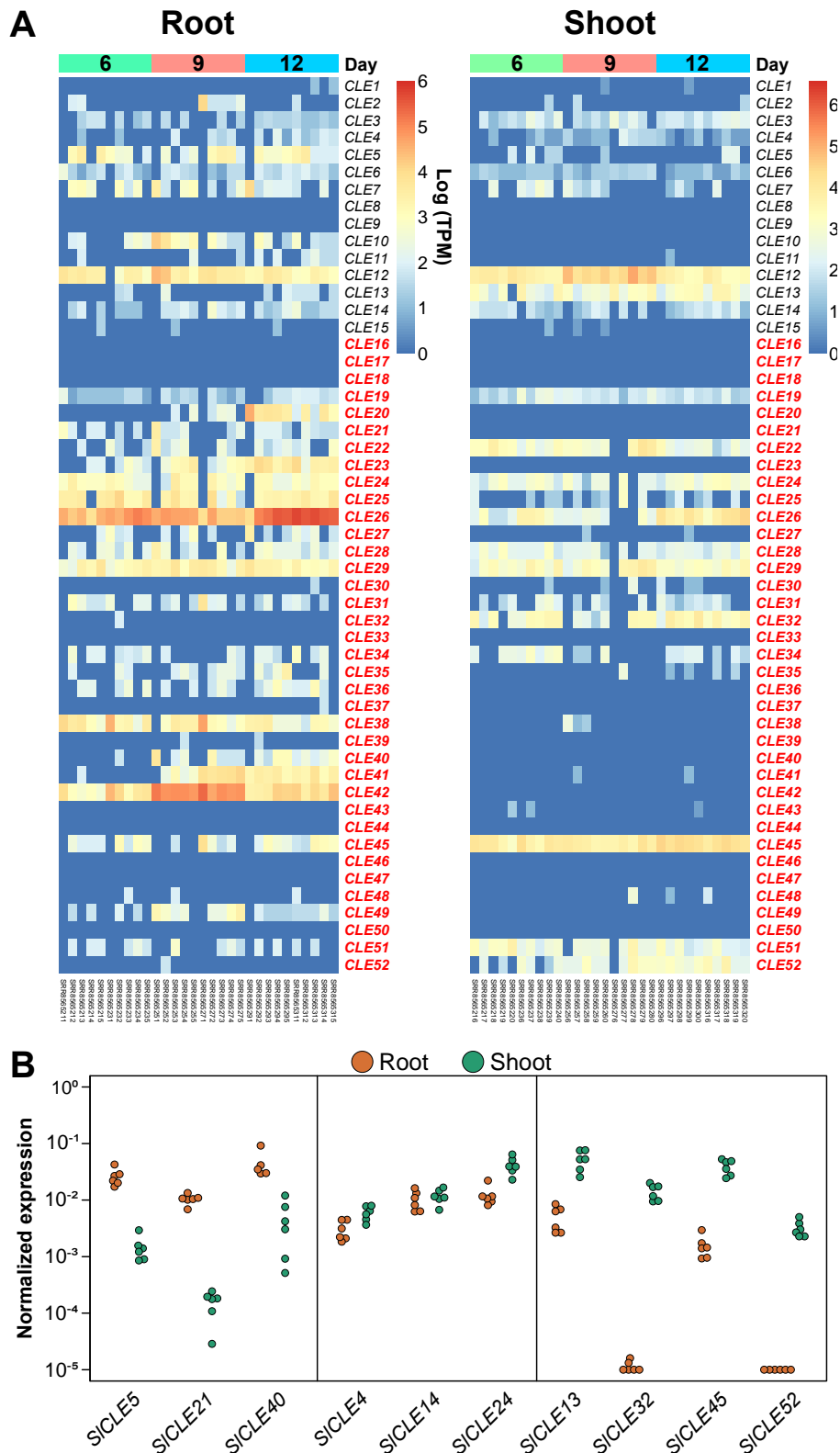
Figure 2. **The phylogenetic tree of the full-length CLE proteins from tomato and Arabidopsis.** The groups of proteins sharing high similarity (clusters) are highlighted by background colors. The names in red indicate the new *CLE* genes uncovered in this study. Nodes supported by bootstrap values superior to 50 are indicated by dots of size proportional to the bootstrap values.

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Figure 3. Expression analysis of *SlCLE* genes in root and shoot tissues [34]. A. Heatmaps of log(TPM) of tomato *CLE* genes in the root (left) and the shoot (right) at 6, 9, and 12 days after plantation from tomato grown in pots. **B.** Expression of selected *SlCLE*s in root and shoot tissues by qPCR from 3 weeks old tomato plant grown in hydroponic conditions.

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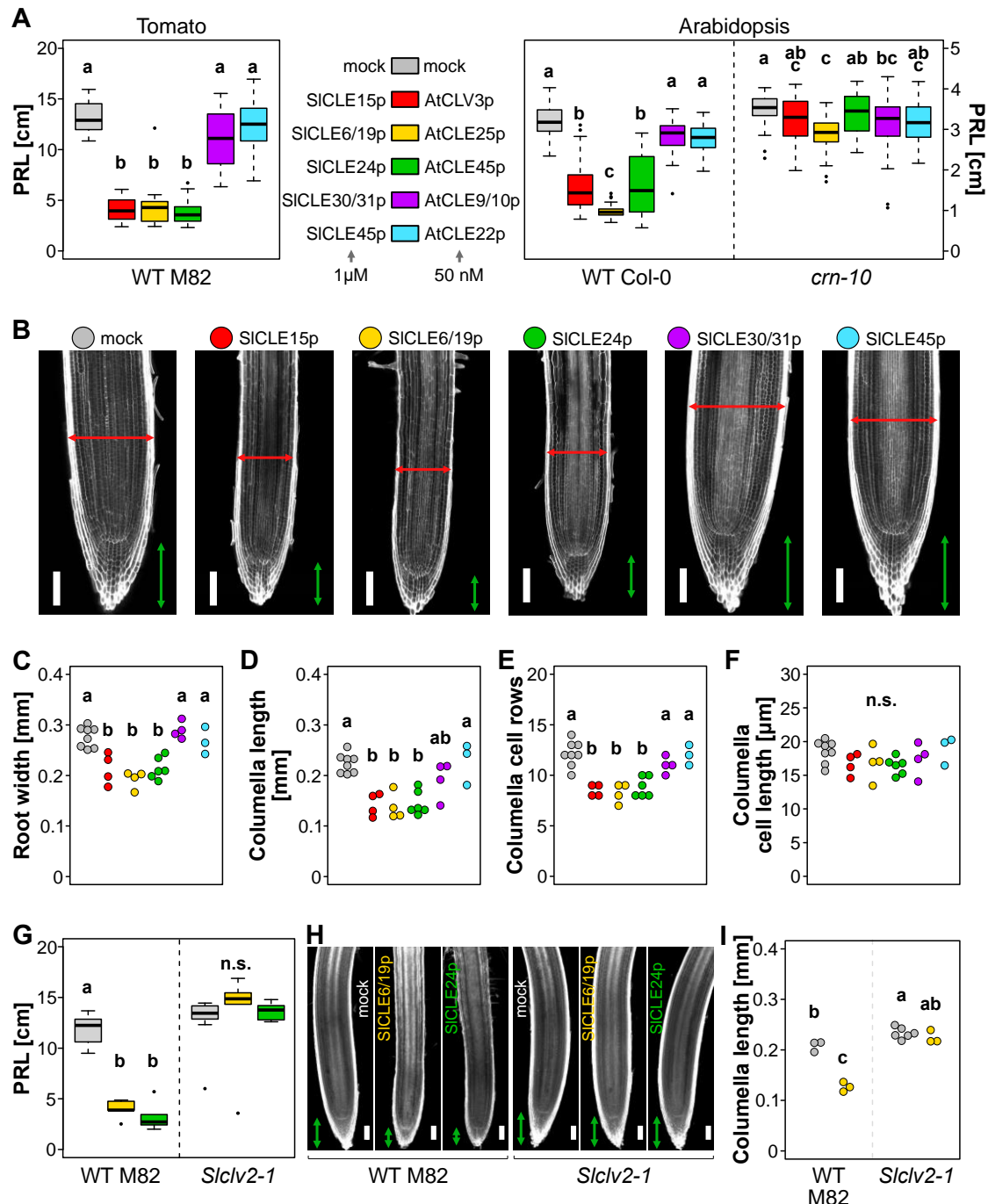
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