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RESEARCH PAPER

Search for nodulation-related *CLE* genes in the genome of *Glycine max*

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Abstract

CLE peptides are potentially involved in nodule organ development and in the autoregulation of nodulation (AON), a systemic process that restricts nodule number. A genome-wide survey of *CLE* peptide genes in the soybean *glycine max* genome resulted in the identification of 39 *GmCLE* genes, the majority of which have not yet been annotated. qRT-PCR analysis indicated two different nodulation-related CLE expression patterns, one linked with nodule primordium development and a new one linked with nodule maturation. Moreover, two *GmCLE* gene pairs, encoding group-III CLE peptides that were previously shown to be involved in AON, had a transient expression pattern during nodule development, were induced by the essential nodulation hormone cytokinin, and one pair was also slightly induced by the addition of nitrate. Hence, our data support the hypothesis that group-III CLE peptides produced in the nodules are involved in primordium homeostasis and intertwined in activating AON, but not in sustaining it.

Key words: Autoregulation of nodulation, CLE peptide, cytokinin, nitrate, nodule primordium.

Introduction

Legumes can grow on nitrogen-poor soils by establishing a symbiosis with soil-borne bacteria called rhizobia. This symbiosis results in the formation of new root organs, the nodules, in which the bacteria fix nitrogen for the plant. In return, the microsymbiont receives carbon sources and a protective niche.

The rhizobia–legume interaction is initiated by mutual recognition of molecular signals. Upon sensing flavonoids exuded by the roots of a compatible host, the rhizobia produce decorated lipochitooligosaccharides, the nodulation (Nod) factors (NFs) that are recognized by LysM receptor-like kinases (RLKs) (D'Haeze and Holsters, 2002). NF recognition activates two co-ordinated plant developmental programmes: initiation of an infection process by which bacteria enter the host and, simultaneously, the elicitation of cortical and pericycle cell division, resulting in the nodule organ. When infection threads reach the cells of the nodule primordium, the bacteria are released into the symbiosomes to fix nitrogen.

Two main nodule types are observed. Determinate nodules, with *Lotus japonicus* as the model legume, are initiated in the outer cortex. Early in development, the primordium cells cease to divide and nodule enlargement is mainly due to cell expansion, resulting in spherical, mature nodules. Indeterminate nodules, for which *Medicago truncatula* (barrel medic) is the model, arise from inner cortical cell division. Some cells of the primordium will become meristematic and will form a persistent apical meristem (Patriarca *et al.*, 2004; Crespi and Frugier, 2008).

Downstream from the NFs, nodule primordium formation depends on cytokinin signalling (Frugier *et al.*, 2008; Oldroyd and Downie, 2008), as demonstrated by knockout mutants for the cytokinin receptor gene *LHK1* in *L. japonicus* or by transgenic *M. truncatula* plants with

reduced expression of the orthologue *CRE1* that were defective in nodule primordia formation (Gonzalez-Rizzo *et al.*, 2006; Murray *et al.*, 2007). In addition, the *L. japonicus snf2* gain-of-function mutant for the LHK1 receptor provoked spontaneous nodules, indicating that cytokinin signalling is both necessary and sufficient for nodule formation (Tirichine *et al.*, 2007). Also auxin flow and signalling are important factors for primordium formation (Mathesius *et al.*, 1998; Boot *et al.*, 1999; Pacios-Bras *et al.*, 2003; van Noorden *et al.*, 2006; Wasson *et al.*, 2006).

Recently, a group of CLAVATA3 (CLV3)/ESR-RELATED (CLE) peptides has been investigated for their role in nodulation (Okamoto *et al.*, 2009, Hirakawa *et al.*, 2010; Mortier *et al.*, 2010). CLE peptides are small (12–13 amino acids) secreted peptides derived from the C-terminal region of pre-proproteins (Mitchum *et al.*, 2008; Oelkers *et al.*, 2008). The *Arabidopsis thaliana* genome contains 32 family members that are involved in balancing proliferation and differentiation during plant development. For instance, in *Arabidopsis*, CLV3 signalling via the RLK CLAVATA1 (CLV1) is essential to maintain stem cell homeostasis at the shoot apical meristem (SAM). Ectopic expression of *CLV3* resulted in the disappearance of the SAM, while *clv3* mutants enhanced SAM proliferation (Clark *et al.*, 1997; Fletcher *et al.*, 1999; Brand *et al.*, 2000; Ogawa *et al.*, 2008). Another well-known example is the CLE41-PHLOEM INTERCALATED WITH XYLEM (PXY) ligand receptor pair that regulates xylem differentiation, and the rate and orientation of vascular cell division (Ito *et al.*, 2006; Hirakawa *et al.*, 2008; Whitford *et al.*, 2008; Etchells and Turner, 2010). CLE peptides with related sequences exhibit functional redundancy (Strabala *et al.*, 2006; Jun *et al.*, 2008). Gain-of-function analysis divided the *Arabidopsis* CLE peptides in at least three groups. Group-I peptides, exemplified by CLV3, arrest premature root and shoot meristem growth when exogenously applied or ectopically produced, indicating that they promote cellular differentiation. Group-II members, exemplified by CLE41, prevent cellular differentiation. No clear function has been described yet for the group-III peptides, comprising the CLE1–CLE7 peptides (Ito *et al.*, 2006; Strabala *et al.*, 2006; Etchells and Turner, 2010).

In *L. japonicus* and *M. truncatula*, three (*LjCLE-RS1*, *LjCLE-RS2*, and *LjCLE3*) and two (*MtCLE12* and *MtCLE13*) *CLE* genes, respectively, are up-regulated during nodulation. The CLE domain of *Lj*CLE-RS1 and *Lj*CLE-RS2 and *Mt*CLE12 and *Mt*CLE13 is highly similar, indicating that these group-III peptides might exert comparable functions (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). In *M. truncatula*, the *MtCLE12* and *MtCLE13* expression patterns suggest a role in primordium and apical meristem homeostasis (Mortier *et al.*, 2010), while functional analysis revealed that *Lj*CLE-RS1, *Lj*CLE-RS2, *Mt*CLE12, and *Mt*CLE13-derived CLE peptides might be implicated in the autoregulation of nodulation (AON) (Okamoto *et al.*, 2009; Mortier *et al.*, 2010).

Long-distance AON signalling controls nodule number to avoid an excess of nitrogen sources that would be deleterious for plant growth (Nutman, 1952). Insight into the process of AON was gained by the isolation of mutants affected in a leucine-rich repeat (LRR)-RLK, designated 'nodule autoregulation receptor kinase' (NARK) or 'nitrogen-tolerant symbiosis 1' (NTS1) in soybean, 'hypernodulation aberrant root formation' (HAR1) in *L. japonicus*, 'symbiosis 29' (SYM29) in pea, and 'super numeric nodules' (SUNN) in *M. truncatula*. They all have a super-nodulation phenotype and exhibit a nitrate-tolerant nodulation, suggesting that the negative control exerted by nitrate on nodulation might happen via the same process (Pierce and Bauer, 1983; Kosslak and Bohlool, 1984; Carroll *et al.*, 1985*a*, *b*; Duc and Messager, 1989; Wopereis *et al.*, 2000; Krusell *et al.*, 2002; Nishimura *et al.*, 2002; Searle *et al.*, 2003; Oka-Kira *et al.*, 2005; Schnabel *et al.*, 2005, 2010; Barbulova *et al.*, 2007; Magori and Kawaguchi, 2009). Grafting experiments have shown that this LRR-RLK of AON is active in the shoot (Nishimura *et al.*, 2002; Searle *et al.*, 2003) and leads to a return signal that is translocated to the roots, to inhibit further nodulation (Nishimura *et al.*, 2002; Lin *et al.*, 2010).

The nature of the AON signalling molecules is still elusive. AON is activated upon NF signalling at the onset of or during cortical cell division (Mathews *et al.*, 1989; Caetano-Anollés and Gresshoff, 1991; Sagan and Gresshoff, 1996; Suzuki *et al.*, 2008; Li *et al.*, 2009) and, in pea and *L. japonicus* become stronger as the nodules matured (Suzuki *et al.*, 2008; Li *et al.*, 2009). SUNN and its orthologues might perceive CLE peptides to triggering AON, because they are phylogenetically related to many known and putative CLE receptors (Shiu and Bleecker, 2001; Okamoto *et al.*, 2009). The *Lj*CLE-RS1, *Lj*CLE-RS2, *Mt*CLE12, and *Mt*CLE13 peptides are good candidates for triggering AON. Indeed, ectopic expression of the corresponding genes strongly reduced or abolished nodulation locally and systemically in a HAR1- and SUNN-dependent way, in *L. japonicus* and *M. truncatula*, respectively (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; V Mortier, unpublished results). Importantly, inhibition of nodulation was specific for overexpression of *LjCLE-RS1*, *LjCLE-RS2*, *MtCLE12*, and *MtCLE13* and ectopic expression of *CLE* genes with a structurally unrelated CLE domain did not induce this effect (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). So far, however, it is not proven that the peptides derived from these genes act as long-distance signals travelling from the developing nodules to the shoot, where SUNN and its orthologues are active.

To gain more insight into the function of CLE peptides in nodulation, *CLE* gene expression was analysed during the development of determinate soybean nodules. Specialized searches predicted 24 peptide genes in the genome of soybean on top of the 15 previously identified (Oelkers *et al.*, 2008). Expression was assayed in various tissues, including developing and mature nodules, after the application of various concentrations of nitrate and of cytokinin and auxin. Several *GmCLE* genes were found of which expression was up-regulated during nodulation. For six *GmCLE* genes, encoding group-I CLE peptides and divided

in three gene pairs, the expression increased steadily during nodulation. Two pairs of *CLE* genes encoded group-III *Gm*CLE peptides. These genes, as well as one group-I *GmCLE* gene, were expressed at high levels in developing, but not in mature, nodules. The group-III soybean genes were induced by cytokinin. These data support the hypothesis that group-III CLE peptides produced in the nodules are involved in primordium homeostasis. These peptides might also activate AON, but not sustain it because genes encoding this group of peptides were absent in mature nodules.

Materials and methods

Plant material, bacterial strains, and growth conditions

Glycine max (L.) Merr. 'Prima 2000' seeds were germinated in the dark for 2 d and grown in pots containing vermiculite (Mandoval, Alberton, South Africa). The greenhouse conditions were 27/17 °C day/night temperature, 60% relative humidity, 13 h photoperiod, 600 mmol m^{-2} s^{-1} photosynthetically active radiation. The plants were watered every 2 d with Hoagland's solution (Hewitt, 1966). For plants grown in nitrogen-rich conditions, NH_4NO_3 (1 mM final concentration) was added to the Hoagland's solution. *Bradyrhizobium japonicum* WB74-1×109 CFU g^{-1} (Soygro biofertilizer Limited, Potchefstroom, South Africa) was inoculated just before sowing by adding 0.5 g of the powder to each pot.

Nodules were harvested 2 and 4 weeks post-inoculation (wpi) for microscopy and expression analysis. For the quantitative reverse-transcription (qRT)-PCR experiments, roots from non-inoculated plants grown under nitrogen-poor conditions (Hoagland's solution) were harvested 2 weeks after sowing. First leaves, cotyledons, and SAMs were harvested 1 week after growing under nitrogen-rich conditions (Hoagland's solution+1 mM NH_4NO_3) and stems, root tips, and leaves 1 week later.

RNA extraction, cDNA synthesis, and qRT-PCR analysis

RNA extraction, cDNA synthesis, and qRT-PCR analysis were done as described by Mortier *et al.* (2010). The relative expression was normalized against the constitutively expressed genes encoding the 40S ribosomal protein S8 (AK285894) or ELF1B protein (TC203623) (Jian *et al.*, 2008). For the five single *GmCLE* genes, specific primer pairs could be predicted by a comparison of the whole soybean genome. For the remaining *GmCLE* genes, no specific regions could be found because of the highly homologous pairs, in which case, the selected primer pairs amplified both homologous genes. The primers used (see Supplementary Table S1 at *JXB* online) were purchased at Inqaba Biotechnical Industries (Pretoria, South Africa). Each experiment was repeated twice with independent biological tissues. Statistical differences were evaluated with ANOVA by means of the GenStat software (http://www.vsni.co.uk/software/genstat/).

In vitro *application of auxins, cytokinins, and nitrogen*

Auxins [10^{-6}M indole-3-acetic acid (IAA)] or cytokinins [10^{-7} M 6-benzylaminopurine (BAP)] were diluted in dimethylsulphoxide and supplemented to the medium of 5-d-old, *in vitro*-grown seedlings. As a control, plants were grown without supplementary hormones. The seedlings were grown in Magenta boxes (6×6×10 cm) on Gelrite agar (Sigma-Aldrich, St Louis, MO, USA) containing Hoagland's solution (Hewitt, 1966) supplemented with 1 mM NH_4NO_3. The plants were cultured in a room at a temperature of 26 °C with a 16 h photoperiod and light intensity of 70 µE m^{-2} s^{-1} light d^{-1}. After 0, 4, 8, and 24 h of incubation, the roots of four plants were harvested and analysed by qRT-PCR. For the *in vitro* application of nitrogen, 0, 1, 5 or 10 mM KNO_3 were added to the medium of 2-d-old seedlings. The roots of six plants of each condition were harvested 6 d later and analysed by qRT-PCR. All experiments were repeated twice with comparable results.

Microscopy

Root nodules were fixed, dehydrated, and embedded with the Technovit 7100 kit (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's instructions, and sectioned with a microtome (Reichert-Jung, Nussloch, Germany). The 3 µm-thick sections were mounted on coated slides (Sigma-Aldrich). For tissue-specific staining, sections were submerged in a 0.5% (w/v) toluidine blue solution, washed in distilled water, and dried. Finally, sections were mounted with Depex (BDH Chemicals, Poole, England). Photographs were taken with a Diaplan microscope equipped with bright-field optics (Leitz, Wetzlar, Germany).

Results

In silico *identification of* GmCLE *genes*

Because CLE pre-proproteins are short and the conserved CLE domain is only 12 amino acids (AAs) long, neither BLAST nor phylogeny could be reliably applied to identify the genes. For that reason, to search for *GmCLE* genes, a pipeline (S Rombauts and Y Van de Peer, unpublished data) was used based on the HMMer software, which is more sensitive and specific than BLAST or PSI-BLAST (Eddy, 2009). As a first step, Hidden Markov Models (HMMs) were constructed that were derived from a multiple alignment, made with MUSCLE (Edgar, 2004), of all known *M. truncatula* CLE proteins. All conserved regions in the alignment (domains) from six AAs onwards were taken into consideration. Subsequently, an orfeome was constructed from the whole Soybean genome and combined with the known *M. truncatula* CLE peptides was screened with the obtained HMMs. The scores for each HMM, received from the HMMer software, were normalized as a function of the length of each individual domain allowing domains to contribute equally. The final scores were ordered in vectors per gene and stored in a matrix. By applying hierarchical clustering (Cluster 3.0; de Hoon *et al.*, 2004) on the matrix, genes with highly correlated vectors were grouped together. The *M. truncatula* CLE peptides included in the analysis pointed to the clusters of interest.

The soybean genes that clustered together with known *M. truncatula CLE* genes were taken as primary candidates. In total, 39 *Gm*CLE proteins were identified, of which 15 had been described previously, but none of them had been annotated in the soybean genome (Oelkers *et al.*, 2008) (Table 1). All hits were annotated and made available at NCBI (Table 1). Due to the duplicated genome of soybean, 17 pairs of highly (at least 85 %) homologous sequences were found.

The corresponding *Gm*CLE pre-proproteins varied in length between 49 AAs and 131 AAs and showed a high level of sequence divergence outside the CLE motif (Table 1). Except for *Gm*CLE13, all proteins had an N-terminal signal peptide or signal anchor as predicted by HMM

Table 1. Overview of the *Gm*CLE peptide genes, the derived CLE domain sequences, identification number, homologous partner, protein length, chromosome number, intron presence, and signal peptide/anchor probability as predicted by HMM signalP and neural networks

G. max nomenclature as defined in this article. General nomenclature is according to Oelkers *et al.* (2008), who numbered each CLE member independently of the species origin and prefixed the numbers with '*CLE*'.

G. max nomenclature	General nomenclature	CLE domain sequence	Identification number	At least 85% homologous to	Length (AA)	Chromosome number	Intron	Signal peptide probability	Signal anchor probability
GmCLE01	*CLE23*	RRVPTGSNPLHN	HM585099	*GmCLE22*	71	14	No	1.000	0.000
GmCLE02	*CLE34*	RRVPNGPDPIHN	HM585100	*GmCLE27*	131	1	No	0.864	0.135
GmCLE03	*CLE51*	HEVPSGPNPISN	HM585101	*GmCLE31*	113	8	No	0.990	0.009
GmCLE04	*CLE52*	RRVPTGPNPLHH	HM585102	*GmCLE24*	111	20	No	0.104	0.890
GmCLE05	*CLE53*	HEVPSGPNPISN	HM585103	*GmCLE26*	123	18	No	0.021	0.968
GmCLE06	*CLE54*	RKVYTGPNPLHN	HM585104	*GmCLE38*	94	19	No	0.998	0.002
GmCLE07	*CLE55*	RRVPSCPDPLHN	HM585105	*GmCLE30*	97	20	No	0.968	0.031
GmCLE08	*CLE56*	RIIHTGPNPLHN	HM585106	*GmCLE28*	114	20	No	0.001	0.999
GmCLE09	*CLE57*	TATPGGPNPLHN	HM585107		86	9	No	0.968	0.032
GmCLE10	*CLE58*	RLVPSGPNPLHN	HM585108		86	10	No	0.985	0.014
GmCLE11	*CLE59*	RKVPNASDPLHN	HM585109	*GmCLE34*	82	14	No	0.265	0.710
GmCLE12	*CLE60*	HEVPSGPNPISN	HM585110	*GmCLE29*	127	15	No	0.999	0.001
GmCLE13	*CLE61*	REVPTGPDPLHH	HM585111		49	12	Yes	0.000	0.000
GmCLE14	*CLE62*	RLAPEGPDPHHN	HM585112	*GmCLE39*	95	13	No	0.974	0.025
GmCLE15		RDVPGGPNPLHN	HM585113	*GmCLE36*	87	5	No	0.995	0.005
GmCLE16		RGVPSGANPLHN	HM585114	*GmCLE33*	67	11	No	0.968	0.032
GmCLE17		REVPSSPDPLHN	HM585115	*GmCLE32*	76	20	No	0.997	0.003
GmCLE18		RIIYTGPNPLHN	HM585116	*GmCLE23*	90	2	No	0.745	0.254
GmCLE19		RLVPSGPNPLHN	HM585117		107	1	No	0.992	0.008
GmCLE20		RLVPTGPNPLHH	HM585118	*GmCLE21*	114	17	No	0.268	0.732
GmCLE21		RLVPTGPNPLHH	HM585119	*GmCLE20*	118	5	No	0.859	0.141
GmCLE22		RRVPTGSNPLHN	HM585120	*GmCLE01*	73	2	No	0.999	0.000
GmCLE23		RIIYTGPNPLHN	HM585121	*GmCLE18*	89	14	No	1.000	0.000
GmCLE24		RRVPTGPNPLHH	HM585122	*GmCLE04*	110	10	No	0.168	0.817
GmCLE25		RRVPTGPNPLHN	HM585123		99	20	No	0.917	0.079
GmCLE26		HEVPSGPNPISN	HM585124	*GmCLE05*	123	8	No	0.018	0.969
GmCLE27		RRVPNGPDPIHN	HM585125	*GmCLE02*	115	2	No	0.959	0.041
GmCLE28		RIIHTGPNPLHN	HM585126	*GmCLE08*	119	7	No	0.001	0.999
GmCLE29		HEVPSGPNPISN	HM585127	*GmCLE12*	125	9	No	0.995	0.004
GmCLE30		RRVPSCPDPLHN	HM585128	*GmCLE07*	96	10	No	0.954	0.045
GmCLE31		HEVPSGPNPISN	HM585129	*GmCLE03*	113	5	No	0.993	0.006
GmCLE32		REVPSSPDPLHN	HM585130	*GmCLE17*	74	10	No	0.999	0.001
GmCLE33		RGVPSGANPLHN	HM585131	*GmCLE16*	67	1	No	0.983	0.016
GmCLE34		RKVPNASDPLHN	HM585132	*GmCLE11*	84	17	No	0.267	0.708
GmCLE35		RLAPGGPDPQHN	HM585133	*GmCLE37*	93	6	No	0.977	0.018
GmCLE36	*CLE63*	RDVPGGPNPLHN	HM585134	*GmCLE15*	87	8	No	0.971	0.027
GmCLE37		RLAPGGPDPQHN	HM585135	*GmCLE35*	94	12	No	0.981	0.018
GmCLE38		RKVYTGPNPLHN	HM585136	*GmCLE06*	100	3	No	0.981	0.019
GmCLE39		RLTPEGPDPHHN	HM585137	*GmCLE14*	96	12	No	0.957	0.041

signalP and neural networks (Bendtsen *et al.*, 2004) (Table 1). Moreover, *GmCLE13* was the only gene bearing an intron (Table 1). The *GmCLE* genes were scattered throughout the chromosomes, except for chromosome 4 and 16 on which no *GmCLE* genes were identified (Table 1). A tree-based alignment was done with the CLE domain encoded by all soybean and *Arabidopsis CLE* genes as well as the nodulation-specific *CLE* genes of *M. truncatula* and *L. japonicus*. Twenty-nine *GmCLE* genes encoded CLE peptides designated as group-I and exemplified by *CLV3* (Fig. 1). Six *GmCLE* genes encoded peptides identical to TDIF/CLE41/CLE44 and were designated as group-II. Four *GmCLE* genes (*GmCLE14*, *GmCLE35*, *GmCLE37*, and *GmCLE39*), encoded peptides that are most homologous to the peptides of the nodulation-specific genes *MtCLE12*, *MtCLE13*, *LjCLE-RS1*, and *LjCLE-RS2* and to *Arabidopsis CLE1* to *CLE7*. This group was named group-III. To facilitate the comparison of the AAs between CLE domains, they were numbered as described by Oelkers *et al.* (2008) with the zero position assigned to the conserved glycine (G) residue located at the centre of the CLE motif and the positions of the other AAs numbered relative to this G. The peptide sequence

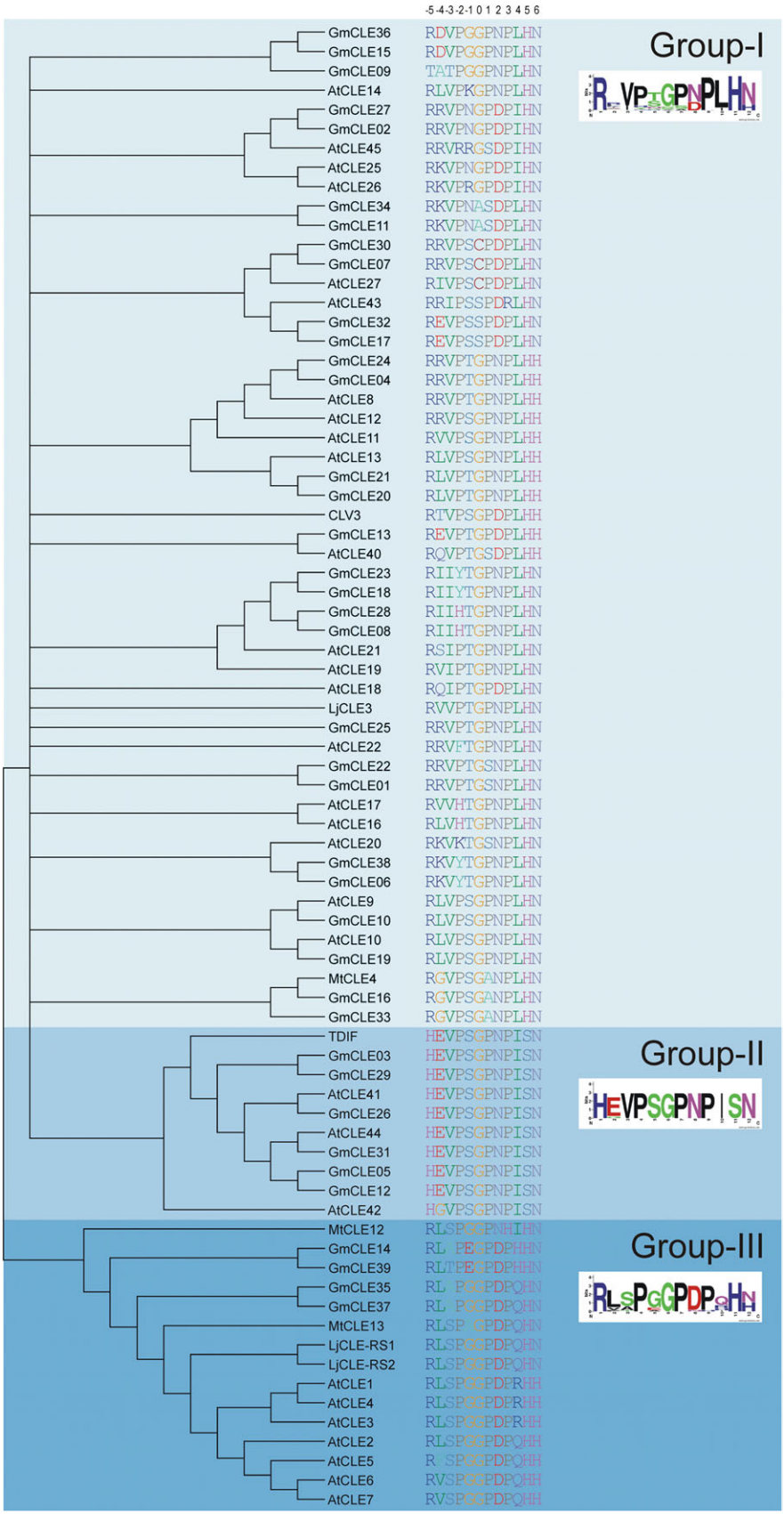

Fig. 1. Tree-based alignment of the CLE domain encoded by all *GmCLE* genes and of the CLE domain of all *Arabidopsis CLE* genes as well as the nodulation-specific *CLE* genes.

derived from the pair *Gm*CLE35-*Gm*CLE37 was very similar to that of the nodulation-related CLE peptides, *Lj*CLE-RS1/*Lj*CLE-RS2 and *Mt*CLE13 (Fig. 1). The sequence only differed at AA positions –3 (A↔S) with *Lj*CLE-RS1/*Lj*CLE-RS2 and –1 (G↔A) and –3 (A↔S) with *Mt*CLE13 (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). By contrast, the CLE domain of *GmCLE14-GmCLE39*, which belongs to the same group-III peptides, differed at least at three AA positions with the other group members. The conserved pattern of the residues in each group is shown in a WebLogo representation (Fig. 1).

Identification of two stages in soybean nodule development with different division and differentiation activities

To identify nodule-related *CLE* genes with expression patterns that are linked with cell division and differentiation, two soybean nodulation stages were analysed, of which one was linked with dividing and differentiating cells and the other corresponded to mature, fully differentiated nodules. At 2 wpi, many small dividing cells were observed at the periphery of a central section through the nodule (Fig. 2A, B). More to the centre, cells differentiated in fixing cells with many infection threads in-between the small cells (Fig. 2A, C). Enlargement showed that many cells in that region were partially filled with symbiosomes, indicating that differentiation is in progress. At 4 wpi, a typical nitrogen fixation tissue was observed consisting of large, infected cells that were completely filled with blue-stained symbiosomes (Fig. 2E, F), and interspersed by vacuolated, uninfected cells (Fig. 2E, F). Cell division or differentiation was no longer observed. To confirm the difference in cell division activity between the two nodulation stages, the expression of the cell division marker, the B-type cyclin *CycB2;1*, was analysed (Umeda *et al.*, 1999; Lee *et al.*, 2003). With BLAST searches, a soybean homologue of the *Arabidopsis AtCycB2;1* was found and was designated *GmCycB2;1*. qRT-PCR was carried out with cDNA derived from both nodulation stages and from uninoculated roots used as a reference tissue. The relative expression of *GmCycB2;1* was higher in the 2-wpi nodule sample than in uninoculated roots (Fig. 3). At 4 wpi, expression was strongly reduced and was even much lower than in uninoculated roots. These results indicate that cell division was high in nodules at 2 wpi and that no cell divisions occurred in mature determinate soybean nodules.

Search for GmCLE *genes that are differentially expressed during nodulation*

To determine the temporal expression during nodulation, qRT-PCR was carried out with cDNA derived from the nodulation-susceptible zone of non-inoculated roots and from nodules at 2 wpi and 4 wpi. The non-inoculated roots were used as reference tissue. With the primer 'Beacon designer 7' program, no primers could be designed that discriminated between the two highly homologous genes of the different *GmCLE* gene pairs. Therefore, primer combinations were used that recognized transcripts of both genes of a single pair. Except for *GmCLE25*, all other 38 *GmCLE* genes were expressed. For eight primer combinations, no differential expression was observed upon nodulation (see Supplementary Table S2 at *JXB* online). Compared with the expression in uninfected roots, the transcript level of *GmCLE06-GmCLE38*, *GmCLE14-GmCLE39*, and *GmCLE35-GmCLE37* increased in nodules at 2 wpi, but decreased again in nodules at 4 wpi (Fig. 4A–C). The

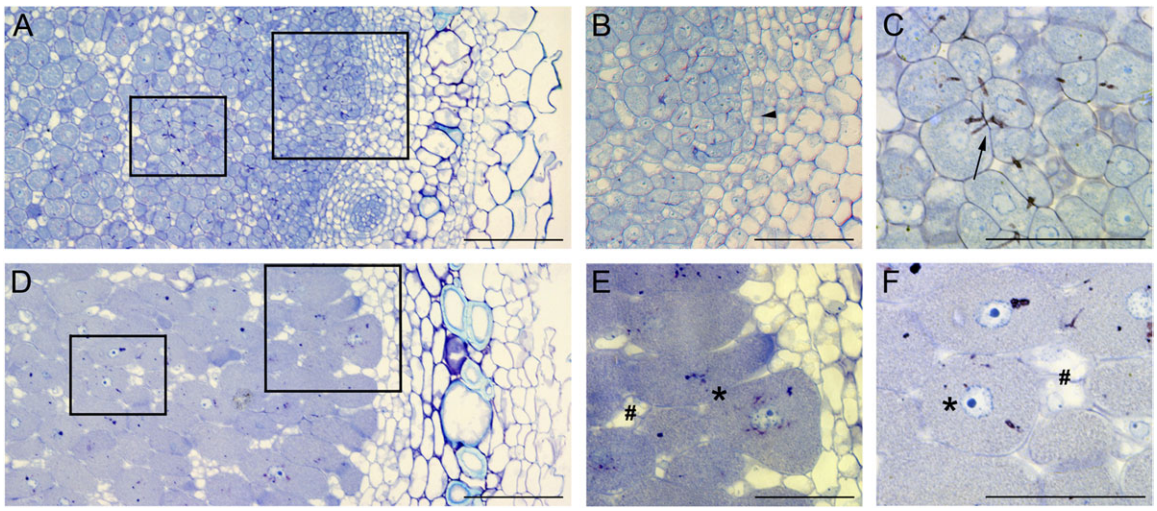

Fig. 2. Microscopic analysis of nodules at 2 wpi and 4 wpi. (A) Section through a nodule of 2 wpi. (B) Detail of large square indicated in (A). Small dividing cells are indicated by an arrowhead. (C) Detail of small square indicated in (A). Differentiation into nitrogen-fixing cells is observed by the presence of many infection threads (arrow). (D) Section through a nodule of 4 wpi. (E, F) Details of (D). Nitrogen fixation zone consisting of large infected cells that are totally filled with symbiosomes (asterisks), interspersed by vacuolated uninfected cells (hashes). No signs of cell division or differentiation are visible anymore. Sections were stained with toluidine blue. Bars: (A, D) 100 μm; (B, C, E, F) 50 μm.

transcript level of *GmCLE11-GmCLE34*, *GmCLE13*, and *GmCLE17-GmCLE32* steadily increased as nodulation progressed and was the highest in mature 4-wpi nodules (Fig. 4D–F). Expression of *GmCLE04-GmCLE24*, *GmCLE09*, *GmCLE12-GmCLE29*, *GmCLE15-GmCLE36*, *GmCLE16-GmCLE33*, *GmCLE18-GmCLE23*, and *GmCLE20-GmCLE21* was much lower in nodules at 2 wpi and 4 wpi than in uninoculated roots (see Supplementary Table S2 at *JXB* online).

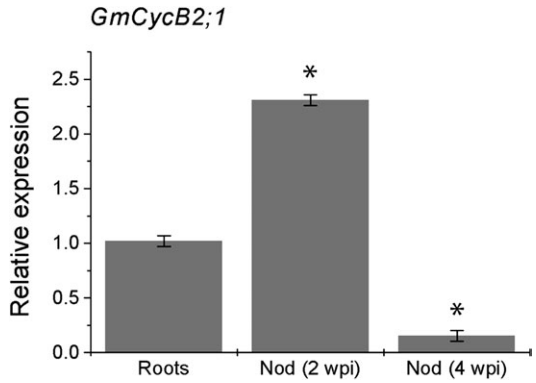

Fig. 3. Expression analysis of *GmCycB2;1* during nodulation. qRT-PCR on cDNA samples of uninoculated roots (Roots) and of nodules (Nod) at 2 and 4 wpi. Statistical differences were evaluated with ANOVA by means of the GenStat software. Error bars represent standard errors (n=2). * Statistically significant differences compared with uninoculated roots (Roots) (P <0.001).

Tissue- or organ-specific expression of nodulation-related CLE peptide genes

GmCLE genes, for which the expression was up-regulated upon nodulation, were also investigated for expression in other tissues or organs. qRT-PCR analysis was carried out with cDNA derived from roots, root tips, stems, SAMs, cotyledons, mature leaves, and first leaves. Expression measured in roots grown without nitrogen was taken as a reference. A basal expression was observed for every *GmCLE* gene in most of the cDNA samples (Fig. 5; see Supplementary Table S3 at *JXB* online). The expression of *GmCLE06-GmCLE38* and *GmCLE13* was higher in the different shoot tissues than in nitrogen-starved roots. Expression of *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37*, both transiently expressed upon nodulation, was higher in roots than in the different shoot tissues (Fig. 5; see Supplementary Table S3 at *JXB* online).

Induction of nodulation-related GmCLE *genes by the addition of auxin or cytokinin*

To see whether the expression of nodulation-related *GmCLE* genes was linked with primordium formation, their expression was assayed after the addition of either auxins or cytokinins, two hormones that control nodule organ formation (Oldroyd and Downie, 2008; Ding and Oldroyd, 2009). Expression of the nodulation-related *GmCLE* genes was assayed in roots of 5-d-old soybean seedlings grown in

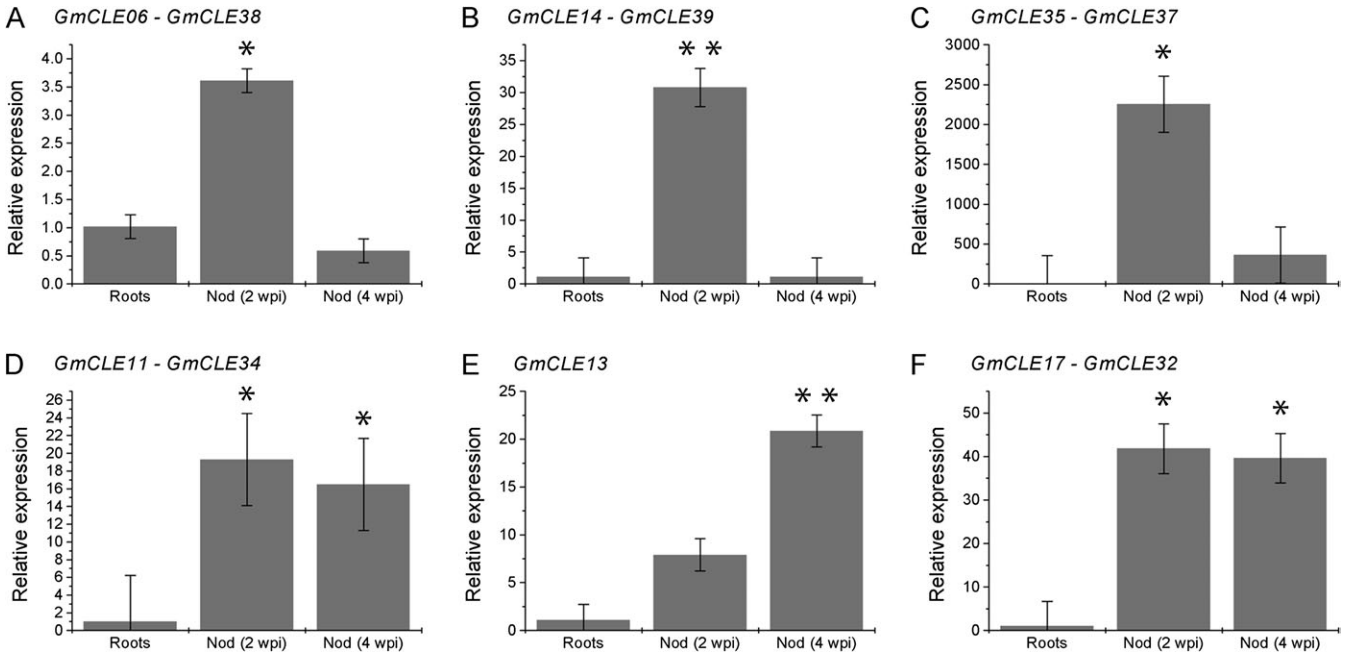

Fig. 4. Expression analysis of *GmCLE* genes during nodulation. qRT-PCR on cDNA samples of uninoculated roots (Roots) and of nodules (Nod) at 2 and 4 wpi. Statistical differences were evaluated with ANOVA by means of the GenStat software. Error bars represent standard errors (n=2). Statistically significant differences compared with uninoculated roots (Roots) are indicated with * (P <0.05) or ** (P <0.01).

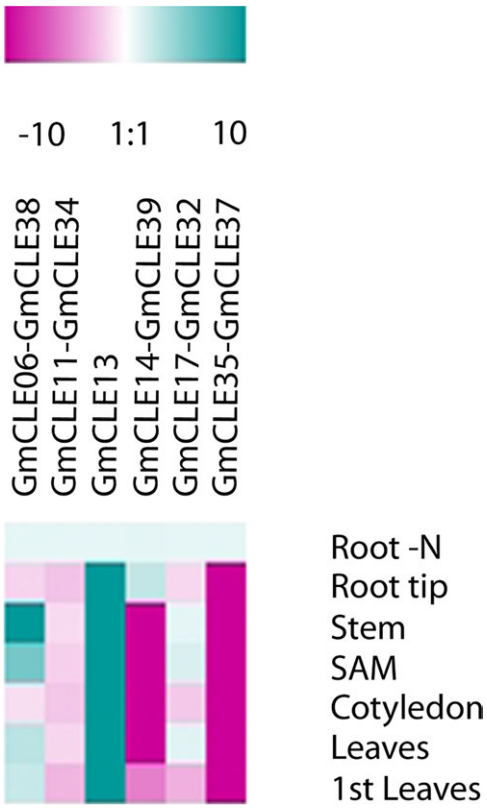

Fig. 5. Tissue- or organ-specific expression analysis of nodulation-related *GmCLE* genes. Heat map of *GmCLE* expression in different tissues as measured by qRT-PCR. Samples are cDNA from roots grown in the absence of NH_4NO_3 (Root –N), root tips, stems, SAMs, cotyledons, mature leaves (leaves), and first leaves (1st leaves).

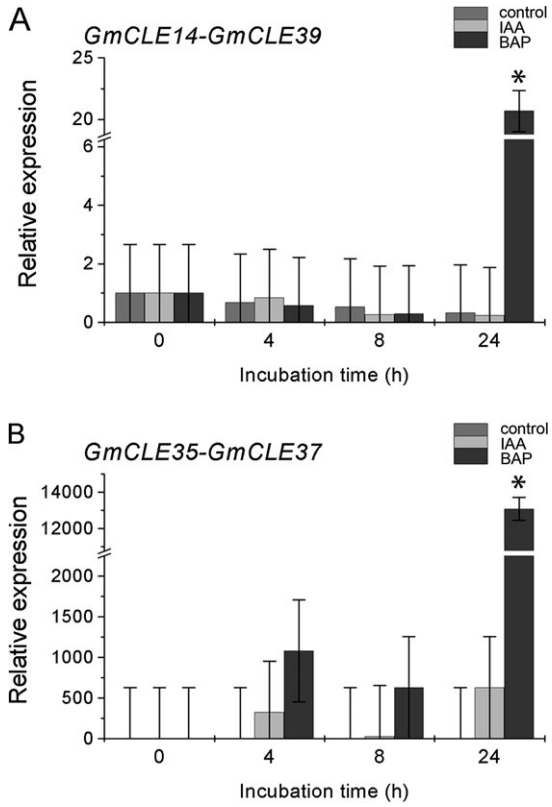

Fig. 6. Influence of auxin and cytokinin on *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* expression. qRT-PCR analysis of *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* expression on cDNA samples of roots grown in the presence of 10^{-6} M auxin (IAA) or 10^{-7} M cytokinin (BAP). Growth medium without hormones was used for the control plants. Samples of 5-d-old plants were taken at 0, 4, 8, and 24 h after hormone addition. Statistical differences were evaluated with ANOVA by means of the GenStat software. Error bars represent standard errors (n=2). * Statistically significant differences in comparison to control plants, grown without hormone addition (P <0.001).

the presence of 10^{-6} M IAA or 10^{-7} M BAP and roots were harvested at 0, 4, 8, and 24 h after treatment. Addition of auxin had no influence on any of the genes tested (see Supplementary Table S4 at *JXB* online; Fig. 6). In samples supplemented with 10^{-7} M BAP, *GmCLE14-GmCLE39*, and *GmCLE35-GmCLE37* transcripts were up-regulated 24 h after treatment (Fig. 6A, B). The expression of the other nodulation-related *GmCLE* genes did not change after BAP treatment (see Supplementary Table S4 at *JXB* online).

Influence of nitrate on the expression of the nodulation-related GmCLE *genes*

Nitrogen starvation is a prerequisite for nodulation and high nitrate availability negatively regulates nodulation (Streeter, 1988; Barbulova *et al.*, 2007). The influence of nitrate on nodulation has been proposed to happen via the AON mechanism because the nodulation of mutants affected in AON is nitrate tolerant (Pierce and Bauer, 1983; Carroll *et al.*, 1985*a*, *b*; Wopereis *et al.*, 2000; Oka-Kira *et al.*, 2005; Barbulova *et al.*, 2007; Magori and Kawaguchi, 2009). To analyse whether nitrate has an influence on the expression of nodulation-related *GmCLE* genes, soybean seedlings were grown for 6 d in the presence of 0, 1, 5 or 10 mM KNO_3. In both biological repeats, the expression of *GmCLE14-GmCLE39* increased after the addition of 10 mM KNO_3 (see Supplementary Table S5 at *JXB* online). However, due to the variation in the level of up-regulation between both repeats, these data were not proven to be statistically different (Fig. 7; see Supplementary Table S5 at *JXB* online). Also for the other genes no statistical difference in gene expression level was seen between control roots and roots treated with the various concentrations of nitrate (see Supplementary Table S5 at *JXB* online).

Discussion

The previously identified *L. japonicus LjCLE-RS1*/*LjCLE-RS2* and the *M. truncatula MtCLE12* and *MtCLE13* genes encode structurally related CLE peptides that are involved in nodulation (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). While tissue-specific expression patterns hinted at a role in nodule primordium and meristem homeostasis, functional analysis revealed a role during AON (Okamoto *et al.*, 2009;

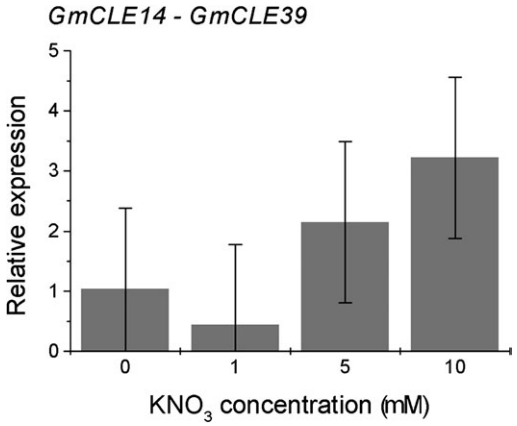

Fig. 7. Influence of KNO_3 on *GmCLE14-GmCLE39* expression. qRT-PCR analysis of *GmCLE14-GmCLE39* expression in cDNA samples of roots grown for 6 d in the presence of 0, 1, 5, and 10 mM KNO_3. Statistical differences were evaluated with ANOVA by means of the GenStat software. No statistical differences were measured as compared to the plants grown in the absence of KNO_3. Error bars represent standard errors (n=2).

Mortier *et al.*, 2010). To get a better insight into the role of CLE peptides in nodulation, the expression of CLE peptide genes was examined during determinate nodule development in soybean, so far the only legume for which the complete genome sequence is available (Schmutz *et al.*, 2010). By specialized searches, 39 *CLE* genes were identified, 34 of which form homologous pairs, as a result of the duplicated genome (Schmutz *et al.*, 2010). Although 15 of these had been previously identified (Oelkers *et al.*, 2008), none of them had been annotated in the genome (Table 1).

By comparing the *Gm*CLE peptide sequences with those of *Arabidopsis*, 29 *Gm*CLE peptides were found to belong to group-I, putative promoters of cellular differentiation exemplified by CLV3 (Ito *et al.*, 2006) (Fig. 1); six *Gm*CLE peptides are related to TDIF and *At*CLE41/*At*CLE44, which are group-II members, known to prevent cellular differentiation and to control the rate and orientation of vascular cell division (Ito *et al.*, 2006; Etchells and Turner, 2010) (Fig. 1). Two *Gm*CLE pairs, *Gm*CLE14-*Gm*CLE39 and *Gm*CLE35-*Gm*CLE37, belong to group III and are similar to the *Arabidopsis* CLE1–CLE7 peptides. In *Arabidopsis,* the functional analysis of group-III peptides resulted in conflicting data. Ectopic addition caused root meristem consumption only at high peptide concentrations and the peptides did not inhibit xylem differentiation in a zinnia (*Zinnia elegans*) cell culture system (Ito *et al.*, 2006). Overexpression of the corresponding genes did not affect the root meristem, while the shoot meristem disappeared (Meng *et al.*, 2010). Also the nodulation-related *Lj*CLE-RS1/*Lj*CLE-RS2, *Mt*CLE12, and *Mt*CLE13 peptides belong to group III (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). Hence, *Gm*CLE14-*Gm*CLE39 and *Gm*CLE35-*Gm*CLE37 might exert a similar function as *Lj*CLE-RS1/*Lj*CLE-RS2, *Mt*CLE12, and *Mt*CLE13.

A nodulation-related function of *Gm*CLE14-*Gm*CLE39 and *Gm*CLE35-*Gm*CLE37 is also suggested by the expression patterns analysed at two different stages of soybean nodule development. Microscopic analysis, confirmed by the expression of a soybean homologue of the G_2-M phase-related marker CycB2;1 (Umeda *et al.*, 1999; Lee *et al.*, 2003) revealed that, under our experimental conditions, the central tissue of 2-wpi nodules contains dividing and differentiating cells, while 4-wpi nodules are terminally differentiated. Expression analysis in these two nodulation stages revealed two different nodulation-related *CLE* patterns. The group-III genes *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* as well as the group-I *GmCLE06-GmCLE38* had a transient expression pattern with the highest expression in 2-wpi nodules, after which the expression disappeared again in mature differentiated nodules. The expression of *GmCLE11-GmCLE34*, *GmCLE13*, and *GmCLE17-GmCLE32* steadily increased as nodulation progressed and was the highest in 4-wpi nodules. Thus, although the expression of six *GmCLE* genes, from which five had a second copy in the genome, are induced during nodulation, only the expression of *GmCLE06-GmCLE38* and the group-III peptide genes *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* was linked with stages of nodule cell division and differentiation in the nodule primordium (Table 2). Compared with *L. japonicus* and *M. truncatula*, more *GmCLE* genes were up-regulated during nodulation of soybean, and different nodulation-related expression patterns were seen. Of course, completion of the genome sequences might reveal more CLE peptides involved in nodulation in *M. truncatula* and *L. japonicus*.

Similar to the previously identified *M. truncatula* group-III gene *MtCLE13* (Mortier *et al.*, 2010), the *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* genes of soybean were expressed in the developing nodule primordium and expression of both pairs is induced in roots by the addition of cytokinins, but not of auxins. *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37* might therefore be not only the structural but also the functional equivalents of *MtCLE13* (Table 2).

The third transiently expressed gene pair, *GmCLE06-GmCLE38*, encodes group-I type peptides that are very similar to *Lj*CLE3 (Okamoto *et al.*, 2009). So far, no localized expression pattern is known for any of the nodulation-related *LjCLE* genes. Our analysis indicates a role for *GmCLE06-GmCLE38* during primordium homeostasis. In contrast to *GmCLE14-GmCLE39* and *GmCLE35-GmCLE37*, no induction of *GmCLE06-GmCLE38* gene expression was found upon cytokinin or auxin addition to roots.

GmCLE11-GmCLE34, *GmCLE13*, and *GmCLE17-GmCLE32*, for which the expression steadily increased during nodulation, encode group-I type peptides from which members are known to cause consumption of the root meristem upon ectopic addition or overexpression. The deduced peptide sequence of *Gm*CLE13 differed only at one position with *At*CLE40. The latter peptide is involved in the organization of the root apical meristem by repressing the WUSCHEL homologue WOX5 (Stahl *et al.*, 2009). The expression of *GmCLE11-GmCLE34*, *GmCLE13*, and

Table 2. Integrated overview of knowledge about nodulation-related CLE peptides

Summary of all data presented here and those obtained by Mortier *et al.* (2010) and Okamoto *et al.* (2009). In bold, supported by expression and functional data; not bold, still hypothetical functions, because of lack of functional data; NA, not analysed.

Genes	Summary						Possible function in nodulation		
	CLE peptide group	Expression in developing nodules	Expression in mature nodules	Cytokinin induction	Auxin induction	KNO_3 induction	AON	Nodule development	Nitrate regulation
GmCLE11-GmCLE34	**I**	**+**	**+**	**−**	**−**	**−**	−	+	−
GmCLE13	**I**	**+**	**+**	**−**	**−**	**−**	−	+	−
GmCLE17-GmCLE32	**I**	**+**	**+**	**−**	**−**	**−**	−	+	−
GmCLE06-GmCLE38	**I**	**+**	**−**	**−**	**−**	**−**	−	+	−
GmCLE14-GmCLE39	**III**	**+**	**−**	**−**	**−**	**+/−**	+	+	+/−
GmCLE35-GmCLE37	**III**	**+**	**−**	**−**	**−**	**−**	+	+	−
MtCLE12	**III**	**+**	**+**	**−**	**−**	**−**	**+**	**+**	−
MtCLE13	**III**	**+**	**+**	**−**	**−**	**−**	**+**	**+**	−
LjCLE-RS1	**III**	**+**	**NA**	**NA**	**NA**	**−**	**+**	**+**	−
LjCLE-RS2	**III**	**+**	**NA**	**NA**	**NA**	**+**	**+**	**+**	+

GmCLE17-GmCLE32 was not modulated by auxin or cytokinin. What the function would be of these CLE peptides in mature, terminally differentiated nodules, without stages of division or differentiation, is not known, but very intriguing because, so far, in all studied cases, no function other than one linked to cell division and differention has been found.

The group-III *Lj*CLE-RS1/*Lj*CLE-RS2, *Mt*CLE12, and *Mt*CLE13 peptides might be involved in AON as overexpression of the corresponding genes reduced or abolished nodulation locally as well as systemically in a SUNN/HAR1-dependent manner (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). This effect was specific for group-III peptides because ectopic expression of *CLE* genes encoding peptides with a typical group-I signature was ineffective (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). The *Lj*CLE-RS1, *Lj*CLE-RS2, *Mt*CLE12, and *Mt*CLE13 peptides could possibly act as long-distance signals and travel to the shoot to be perceived by SUNN/HAR1 (Okamoto *et al.*, 2009; Mortier *et al.*, 2010). Alternatively, in nodules, the group-III peptides might be perceived by local receptors and provoke an upward long-distance signal that activates, in the shoot, the binding of an, as yet unidentified, CLE peptide with SUNN/HAR1 for AON.

The soybean group-III peptides *Gm*CLE14-*Gm*CLE39 and *Gm*CLE35-*Gm*CLE37 might, based on the sequence similarity and the expression analysis, be the functional equivalents of *Lj*CLE-RS1, *Lj*CLE-RS2, *Mt*CLE12, and/or *Mt*CLE13 (Table 2) and activate AON in soybean. Interestingly, because these soybean group-III *CLE* genes are not expressed in mature nodules, which exert a high AON activity, our data open the possibility that group-III CLE peptides might activate AON, while other mechanisms take over the control of nodule numbers at later stages during nodulation, possibly involving nitrogen fixation efficiency (Nutman, 1952; Magori and Kawaguchi, 2009).

Mutants defective in AON, exhibit a nitrate-tolerant nodulation, suggesting that nitrate inhibition of nodulation act via the AON pathway (Pierce and Bauer, 1983; Carroll *et al.*, 1985*a*, *b*; Wopereis *et al.*, 2000; Oka-Kira *et al.*, 2005; Barbulova *et al.*, 2007; Magori and Kawaguchi, 2009). In *L. japonicus*, *Lj*CLE-RS2 transcription was up-regulated by nitrate (Okamoto *et al.*, 2009). Under our experimental conditions, the expression of one group-III *GmCLE* gene pair was moderately induced by the addition of nitrate (Table 2). Hence, the link between AON, CLE peptides and the influence of nitrate on nodule formation needs further investigation.

In conclusion, in soybean, several CLE genes are up-regulated during nodulation. Two distinct nodulation-related expression patterns were observed, one linked with nodule primordium formation and differentiation and another linked with nodule maturation. It would be interesting to follow the localized expression of these *CLE* genes and to study the effects of knockout or overexpression. Such studies might be hampered by redundancy because, in the soybean genome, CLE peptides are mostly encoded by gene pairs. Expression of group-III gene pairs was linked with developing and not with mature nodules, suggesting that signalling by group-III CLE peptides might initiate AON and that other mechanisms might take over at later stages in nodulation. Our data provide a framework

for biochemical and genetic analysis to explore potential interaction with the SUNN/HAR1/NARK receptor and a role in nodule primordium and meristem homeostasis.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Primers used in the analysis.

Supplementary Table S2. Expression analysis of all *GmCLE* genes during nodulation.

Supplementary Table S3. Tissue- or organ-specific expression analysis of nodulation-related *GmCLE* genes.

Supplementary Table S4. Influence of auxin and cytokinin on the expression of *GmCLE* genes.

Supplementary Table S5. Influence of KNO_3 on the expression of *GmCLE* genes.

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