Petrenko V. A.,
Saint Petersburg University
st106188@student.spbu.ru

Lebedeva M. A.,
Saint Petersburg University
mary_osipova@mail.ru

Lutova L. A.,
Saint Petersburg University
la.lutova@gmail.com

CLE PEPTIDES – KEY NEGATIVE REGULATORS OF NODULATION IN *MEDICAGO TRUNCATULA*

Abstract. This investigation into the Autoregulation of Nodulation (AON) in *Medicago truncatula* characterizes CLE peptides as systemic negative regulators. It confirms the role of known peptides (MtCLE12/13) and identifies MtCLE35 as a novel nitrate– and rhizobia–induced inhibitor. Overexpression of MtCLE35 abolishes nodulation, alters symbiotic gene expression, and induces defense and redox–related genes. The study also proposes MtCLE29and MtCLE14 as new AON candidates.

Key words*:* CLE peptide, AON, nodule formation, defense mechanism

Plants of the legume family have developed a unique adaptation mechanism to nitrogen deficiency—symbiosis with nitrogen–fixing bacteria *Rhizobium*. Within this mutually beneficial cooperation, the bacteria convert molecular nitrogen into absorbable ammonia, while the host plant provides them with carbohydrates and a protected bacterium in specialized organs called nodules, which form on the roots. Since the processes of nodule development and nitrogen fixation requires energy expense, the number of nodules in legumes is strictly controlled. A key mechanism preventing the formation of excessive nodules is the autoregulation of nodulation (AON) system [1, p 352]. Research on AON is currently actively ongoing.

Regulatory peptides of the CLE family are crucial mobile signals in the AON pathway. Synthesized in roots upon rhizobial infection, these peptides are transported to the shoot via the xylem. Their gene expression is induced early in nodule development, and their overexpression potently suppresses nodule formation [2, p 226].

According to the scientific data, 52 genes encoding CLE peptides have been identified in the genome of the model legume *Medicago truncatula*. Of these, only two – *MtCLE12* and *MtCLE13* – have been shown to have their expression activated in response to rhizobial inoculation. The implementation of the autoregulation of nodulation (AON) signaling pathway depends on the interaction of these peptides with the SUNN receptor kinase, which is localized in the plant shoot [3, p. 341].

In *M. truncatula*, activation of *MtCLE13* expression is observed at the earliest stages of symbiosis development – 6 hours after inoculation, while *MtCLE12* expression increases slightly later – 48 hours after inoculation. The *MtCLE13* promoter is active in proliferating cells of the cortex and pericycle, as well as in the apical part of mature nodules. Promoter activity for the *MtCLE12* gene was noted at later stages of nodule development – in the cells of the already formed nodule primordium. Interestingly, the expression of *MtCLE12* and *MtCLE13* genes is

induced by cytokinin but not by nitrate, indicating a complex nodulation regulatory system involving CLE peptides, phytohormones and external stimuli.

In addition to rhizobia–induced CLE peptides, nitrate–induced CLE also regulate nodulation. In *Medicago truncatula*, MtCLE34 is activated by both nitrate and rhizobial inoculation. To test whether MtCLE34 inhibits nodulation like its close homologs in other legumes, it was created an overexpression vector containing the coding sequence from the R108 line, along with a GFP cassette for root selection.However, overexpression of *MtCLE34* did not affect nodule number because the gene contains a premature stop codon in several M. truncatula accessions, including the reference genotype A17, rendering the peptide non–functional [4, p. 842]. Our laboratory has identified a novel peptide, MtCLE35, which also functions as a negative regulator of symbiotic nodule development. Overexpression of the *MtCLE35* gene suppresses the nodulation program. Furthermore, *MtCLE35*expression is induced not only by rhizobial inoculation but also by nitrate treatment.

To elucidate the mechanism by which MtCLE35 suppresses nodulation, we performed a transcriptomic analysis of transgenic *Medicago truncatula* roots overexpressing *MtCLE35* at 11 days post–inoculation with rhizobia. Roots overexpressing the β–glucuronidase (*GUS*) were used as a control. The results demonstrate that overexpression of *MtCLE35* leads to the suppression of 1,122 genes involved in the symbiotic program and a complete absence of nodule formation.

The transcriptome analysis also revealed that the expression levels of certain genes were upregulated in the MtCLE35–overexpressing roots. According to MACE–Seq and subsequent qPCR analysis of transgenic p35S:MtCLE35 roots inoculated with rhizobia, genes associated with reactive oxygen species production and the antioxidant system were induced in the inoculated roots overexpressing *MtCLE35*. These included thioredoxin H2 (TRX), peroxidase 100, and a gene encoding ascorbate oxidase (ACO). Multiple genes encoding cysteine–rich peptides, which are too activated in rhizobia–inoculated roots overexpressing *MtCLE35*, may be involved in redox homeostasis alongside thioredoxins and peroxidases [5, p. 4951]. This is due to the susceptibility of the mercapto (–SH) groups of Cys residues to oxidation. Moreover, since groups of Cys–rich peptides have been reported to possess antimicrobial activity and induce plant defense responses, it could be predicted that these cysteine–rich peptide–encoding genes are part of defense mechanisms (fig. 1).

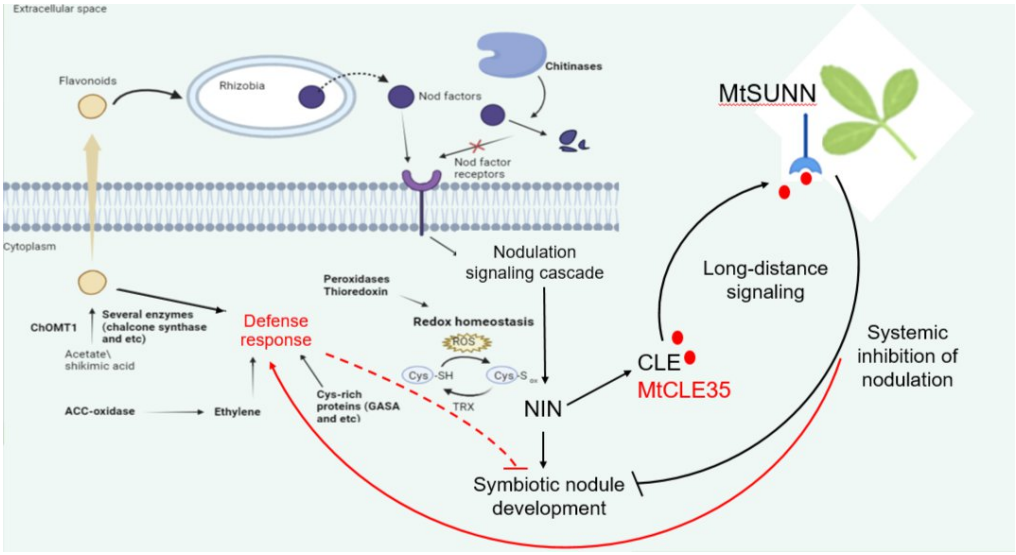

Fig. 1. The scheme illustrating the possible mechanisms underlying inhibition of nodulation program by MtCLE35–induced signaling pathway

To identify new potential participants in the autoregulation of nodulation (AON) system among CLE family peptides, we constructed a phylogenetic tree based on an alignment of the amino acid sequences of the C–terminal CLE domain. Our analysis revealed the peptide MtCLE29, which is related to other known CLE peptides that regulate symbiotic nodule development. The CLE domains of the MtCLE29 and MtCLE35 peptides differ by only one amino acid (arginine in MtCLE29 and histidine in MtCLE35 in the 10–th position of the CLE domain).

According to transcriptomic data from RNA sequencing (MtExpress V3, NCBI BioProject PRJNA391316), MtCLE29 expression increases in developing nodules, a finding we confirmed in our own quantitative PCR (qPCR) experiments. Since CLE35 is known to be nitrate–regulated, we investigated whether the expression of the related CLE29 gene is similarly affected. qPCR data demonstrate that, like *CLE12* and *CLE13, CLE29* expression is not regulated by nitrate (Fig. 2).

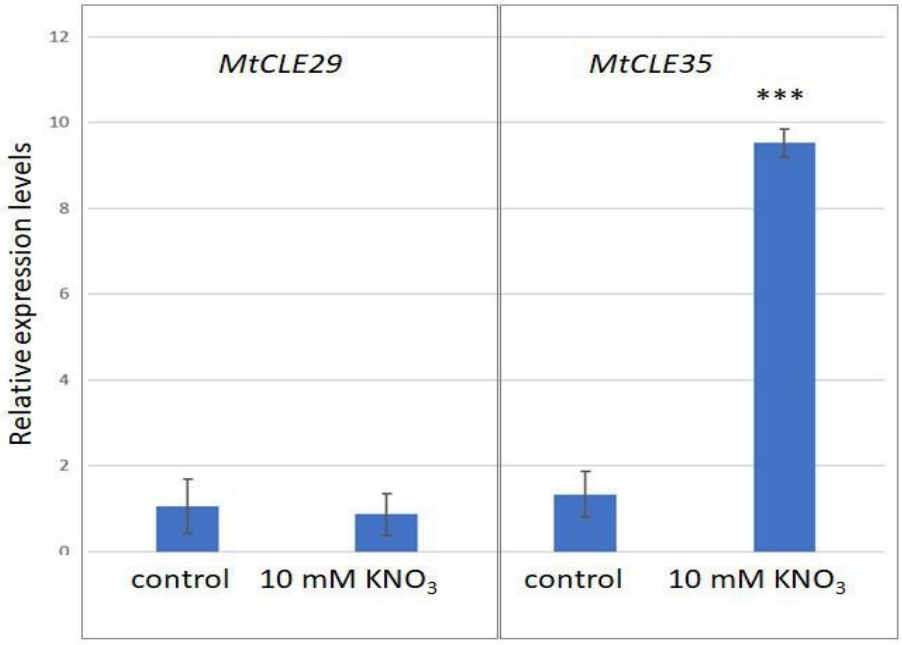

Fig. 2. The levels of *CLE29* and *CLE35* genes after nitrate treatment

It is noteworthy that multidomain CLE peptides have been identified in plants such as *Oryza sativa* and *Arabidopsis thaliana*. In *Medicago truncatula*, we identified a multidomain peptide, CLE14. Analysis of its amino acid sequence revealed that MtCLE14 contains seven CLE domains, four of which are identical to the CLE domain of MtCLE35.

As these peptides have not been previously characterized, we generated overexpression vectors for the *MtCLE29* and *MtCLE14* genes. Transgenic plants have been produced and are currently growing. Subsequent experiments will be conducted to determine the role of these proteins in the systemic regulation of nodulation.

References

1. Caetano–Anollés G., Gresshoff P.M. PLANT GENETIC CONTROL OF NODULATION / G. Caetano–Anollés, P.M. Gresshoff // Annu. Rev. Microbiol. 1991. Т. 45, № 1. С. 345–382.

2. Mortier V. и др. CLE Peptides Control Medicago truncatula Nodulation Locally and Systemically / V. Mortier и др. // Plant Physiology. 2010. Т. 153, № 1. С. 222–237.

3. Mortier V. et al. Nodule numbers are governed by interaction between CLE peptides and cytokinin

signaling / V. Mortier и др. // The Plant Journal. 2012. Т. 70, № 3. С. 367–376.

4. Lebedeva M., Dvornikova K., Lutova L. Nitrate–Induced MtCLE34 Gene Lacks the Ability to Reduce Symbiotic Nodule Number and Carries Nonsense Mutation in a Few Accessions of Medicago truncatula / M. Lebedeva, K. Dvornikova, L. Lutova // Agronomy. 2022. Т. 12, № 4. С. 842.

5. Lebedeva M.A. et al. MtCLE35 Mediates Inhibition of Rhizobia–Induced Signaling Pathway and Upregulation of Defense–Related Genes in Rhizobia–Inoculated Medicago truncatula Roots / M.A. Lebedeva и др. // J Plant Growth Regul. 2024. Т. 43, № 12. С. 4941–4956.