

Systemic control of legume-rhizobia symbiosis: functional studies of key regulators using overexpression and CRISPR/Cas9-mediated gene editing approaches

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Motivation and aim: Legumes enter into symbiosis with soil bacteria rhizobia to satisfy their nitrogen demands: they form symbiotic nodules on the roots, where rhizobia fix atmospheric nitrogen to make it available for plants. Since symbiotic nodule development and maintenance is energy-consuming process, host plants regulate the number of symbiotic nodules depending on the nitrogen needs of the whole organism – at the systemic level. CLE peptides are key players of systemic control of legume-rhizobia symbiosis. CLE peptides are produced in the root in response to nodulation and are capable of long-distance transport along the xylem to the shoot, where, when binding to specific receptors, these peptides trigger a signaling response that suppresses further development of symbiotic nodules on the roots. Our research focuses on the role of MtCLE35 in systemic control of nodulation in *Medicago truncatula*, as well as two target genes activated by MtCLE35-induced signaling pathway: the *MtTML1* (*TOO MUCH LOVE 1*) and *MtTML2* genes.

Materials and methods: We used overexpression and CRISPR/Cas9-mediated gene editing approaches to study the role of MtCLE35 and *MtTMLs* genes in symbiotic nodulation. For *MtCLE35*-overexpressing roots, we performed transcriptomic study to find out mechanism of MtCLE35-mediated inhibition of nodulation. Several lines with *MtCLE35* and *MtTMLs* gene knock-out have been obtained via CRISPR/Cas9-mediated gene editing.

Results: Expression of *MtCLE35* increased both in response to rhizobia and nitrate treatment. Overexpression of *MtCLE35* completely blocked symbiosis with rhizobia. According to transcriptomic and qPCR data, MtCLE35-mediated inhibition of nodulation was accompanied by increased expression levels of defense-related genes in *MtCLE35*-overexpressing roots. Moreover, the *MtTML1* and *MtTML2* genes were upregulated in *MtCLE35*-overexpressing roots. TMLs are known to encode F-box-proteins acting as inhibitors of nodulation. However, their targets have not been found yet. We generated several lines with CRISPR/Cas9-mediated *MtCLE35* gene knock-out, and found that mutant lines with loss of *MtCLE35* function demonstrated increased number of nodules in comparison to wildtype line, when grown in the presence of nitrate. Moreover, we obtained lines with *TMLs* knock-out, which also had increased number of symbiotic nodules.

Conclusions: Therefore, our data demonstrate that MtCLE35 regulates number of symbiotic nodules systemically and mediates nitrate-dependent inhibition of nodulation. MtCLE35-induced signaling pathway affects early steps of legume-rhizobia symbiosis, resulting in enhanced defense response in rhizobia-inoculated roots. *TMLs* transcripts are accumulated in response to MtCLE35-induced signaling pathway. Several lines with *MtCLE35* and *MtTMLs* gene knock-out have been obtained via CRISPR/Cas9-mediated gene editing, which form increased number of symbiotic nodules. Therefore, *CLE* and *TML* genes are good candidates for CRISPR/Cas9-mediated gene editing in order to increase symbiotic efficiency in agronomically important legumes, which, in its turn, should help to increase protein yield and overall productivity in legume crops.