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The involvement of root-specific LTPs in the symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti*

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Lipid transfer proteins (LTPs) are small basic proteins that constitute a large family characterized by the ability to transfer phospholipids between a donor and an acceptor membrane and can have many different roles *in vivo*. Recently it has been demonstrated that MtN5, a non specific LTP (ns-LTP) classified as type III (Wang *et al*., 2012), is involved in the symbiotic interaction between legumes and rhizobia (Pii *et al.* 2009, Pii *et al*., 2012). *MtN5* is a nod factor responsive gene expressed at a very early phase of rhizobial symbiosis in the epidermis and root hairs and later in primordia and nodules. There are evidences that *MtN5* positively regulates the nodulation process. Interestingly, two other putative type III ns-LTPs (*Medtr3g055250* and *Medtr7g052640*) have been identified in *Medicago truncatula* genome. The aim of this study is to shed light on the role of these ns-LTPs in the symbiotic interaction between *M. truncatula* and *Sinorhizobium meliloti.*

The early nodulin MtN5 is required for optimal bacterial infection and nodule invasion

In nodulated plants, the **MtN5 gene is highly expressed in the root nodules**, where the level of expression is 7fold higher with respect to the transcript level of non nodulated roots or nodulated roots deprived of nodules (Fig. 1).

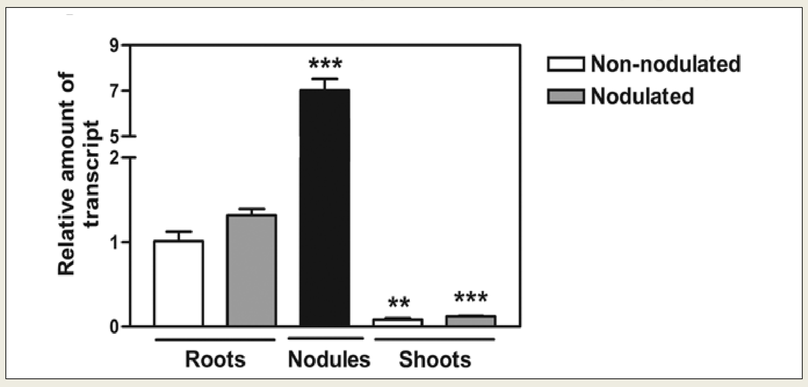

*Fig. 1 – Expression of the M. truncatula N5 gene in roots and shoots of both non nodulated and nodulated plants and in nodules. The values reported are means ± standard error (** P < 0.01; *** P < 0.001) (Pii et al. 2009).*

MtN5 protein is detectable in inoculated roots starting from 1 dpi and it reaches the highest concentration at 3 dpi (Fig. 2). These data suggest that MtN5 induction is an early event that might occur before invasion of the root by rhizobia.

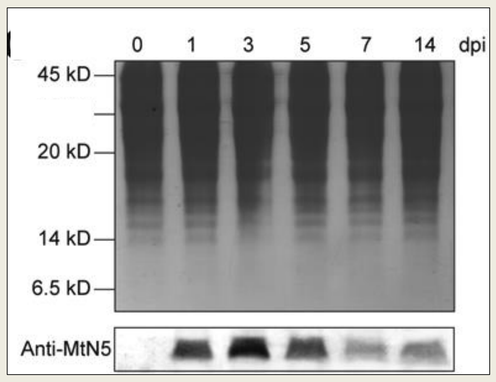

Fig. 2 – Western blot analysis of proteins extracted from root apparatuses after microflood inoculation with Sinorhizobium meliloti. Roots were collected 1, 3, 5, 7, and 14 days post inoculation (Pii et al. 2009).

In ***MtN5p::GUS*** transgenic roots GUS activity is visible **in the root hairs** 3 hours post-inoculation (Fig. 3A). At more advanced stages of infection (24 hpi), the promoter activity is detected **in the root cortex**, (Fig. 3B) and in **nodule primordia** (Fig. 3C).

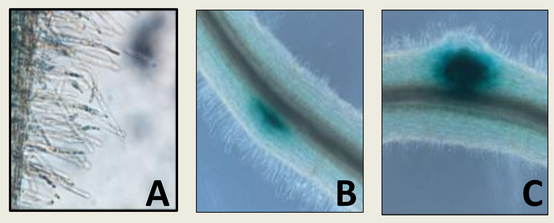

Fig. 3 - Localization of MtN5 expression in the root hairs at 3 hpi (A), in the root cortex at 24 hpi (B) and in nodule primordia (C) (Pii et al. 2012).

MtN5-silenced roots inoculated with rhizobia display an increased root hair curling and a reduced number of invaded primordia compared to that in wild type roots, suggesting a possible role in bacterial infection and nodule invasion.

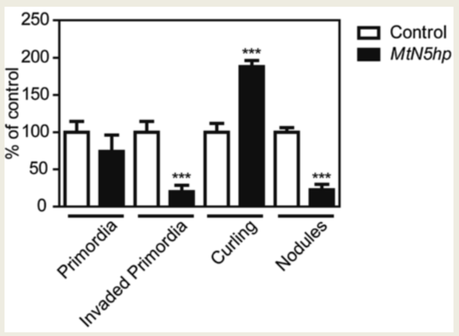

*Fig. 4 – Number of root hair curling events, total and invaded primordia and mature nodules in MtN5hp roots inoculated with S. meliloti. The data reported are means ± SE (n=40) calculated as percentage relatively to control inoculated roots. Student's t test was applied. *** P < 0.001 (Pii et al. 2012).*

We obtained stably transformed plants expressing two different constructs: a hairpin (hp) gene construct designed to silence *MtN5* and a *35S*::*MtN5* construct to overexpress the gene. As shown in Fig. 5, ***MtN5*-silenced plants are impaired in nodulation**, showing a 40% of reduction in the number of nodules compared with wild type *M. truncatula*. Transgenic **plants overexpressing *MtN5* develope 34% more nodules with respect to control ones**, thus confirming the important role of this LTP in the establishment of the symbiosis.

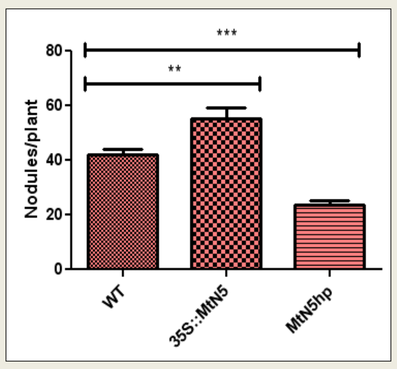

*Fig. 5 – Number of mature nodules in MtN5hp, 35S::MtN5 transgenic and WT plants inoculated with S. meliloti. The values reported are means ± standard error (** P < 0.01; *** P < 0.001)*

Medtr3g055250 and *Medtr7g052640* expression pattern during nodule development

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MtN5           VQICNIDPNDLKQSCSKFVTGRNPPRADEACCGVLRRANLPCLCGYKS--ALTYYGINAKKALALPGQCGLQTPSNC--- 75
Medtr3g055250  IIVCSIDTN-KLDVCHDAITGKRPPKPTTKCCALIKKADLSCLCRYKS--LLPALGINPTKALALPKKCGRKTPPGCRAN 77
Medtr7g052640  MSLCNMNED-GLDACKPSVTQPYPAKPSTECCKALTGADLQCLCSYKNSAELPLLGIDPTLAASLPKECDLTPPSNC--- 76
               : :*.:: :   : *   :*   *.:.   **  :  *:* *** **.   *.  **:. * :** :*.  .*..*
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Fig. 6 – The amino acid sequences of mature MtN5 and two other type III ns-LTPs identified in the M. truncatula genome, aligned using the ClustalW2 program.

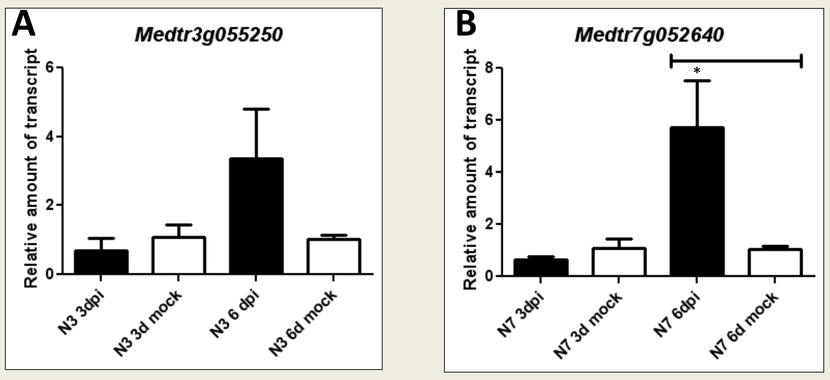

Fig. 7 – Expression level of Medtr3g055250 (A) and Medtr7g052640 (B) genes in M. truncatula 3 and 6 dpi in plants inoculated with S. meliloti and mock-inoculated ones. The values reported are means ± standard error (P < 0.05).*

The expression pattern of the other two type III LTPs was evaluated in *M. truncatula* plants by qRT-PCR. At 3 days post inoculation (3 dpi) no significant differences in the expression levels of the two genes was detected in infected plants in comparison with non-inoculated ones. At **6 dpi the expression of *Medtr7g052640* is significantly increased with respect to the transcript level detected in non-nodulated roots** (Fig. 7B).

***Medtr3g055250* and *Medtr7g052640* promoters activity** was monitored in transgenic roots with and without rhizobial inoculation by means of a reporter gene construct. In non-inoculated plants, GUS activity was detected at the site of lateral root emergence and along the whole length of young lateral roots (data not shown). Both *Medtr7g052640::GUS* and *Medtr3g055250::GUS* transgenic roots showed localized induction of GUS activity in the nodules after *S. meliloti* inoculation. At **18 dpi the expression of the GUS reporter is detectable in the whole nodule**, **predominantly localized in the distal zone** (Fig. 7A and 7B).

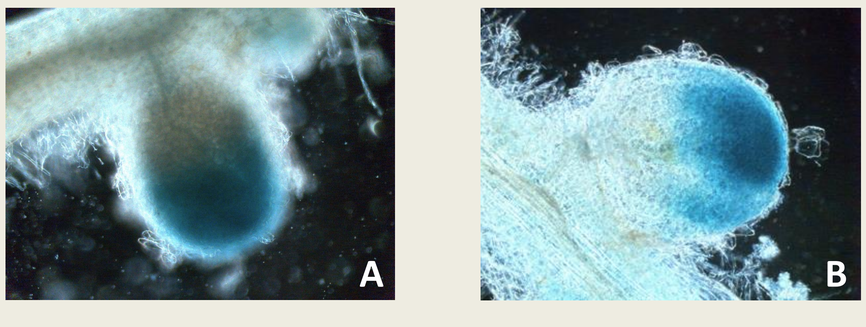

Fig. 8 – Medtr3g055250 (A) and Medtr7g052640 (B) promoter activity in fully developed root nodules (18 dpi).

These data indicate that *Medtr7g052640* and *Medtr3g055250* expression is induced after invasion of the root by rhizobia and suggest a possible involvement of these LTPs in the symbiotic interaction.

MtN5, Medtr3g055250 and Medtr7g052640 expression in mycorrhiza-infected roots

AMF and rhizobia both produce LCO signaling molecules that can activate a common symbiosis signalling pathway. To assess whether these type III LTPs are involved also in the mycorrhizal infection, a quantitative RT-PCR was performed on mycorrhiza-infected roots. **Preliminary data indicate that the expression level of the three genes don't vary, suggesting that these LTPs are specifically involved in the rhizobial symbiosis.**

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