

Chiara Santi¹, Barbara Molesini¹, Youry Pii², Tiziana Pandolfini¹

¹ Department of Biotechnology, University of Verona, Italy

² Faculty of Science and Technology, University of Bolzano, Italy

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).

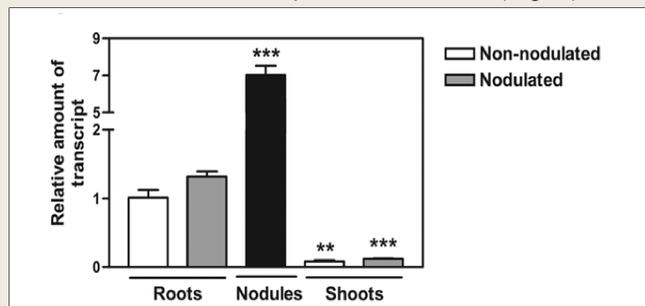


Fig. 1 – Expression of the *M. truncatula* N5 gene in roots and shoots of both non nodulated and nodulated plants and in nodules. The values reported are means \pm standard error (** $P < 0.01$; *** $P < 0.001$) (Pii *et al.* 2009).

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.

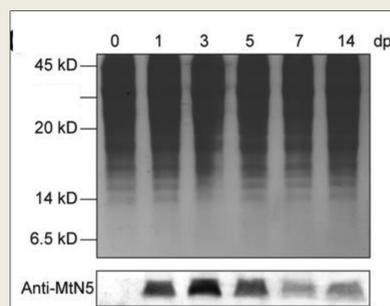


Fig. 2 – Western blot analysis of proteins extracted from root apparatuses after microfluid inoculation with *Sinorhizobium meliloti*. Roots were collected 1, 3, 5, 7, and 14 days post inoculation (Pii *et al.* 2009).

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).

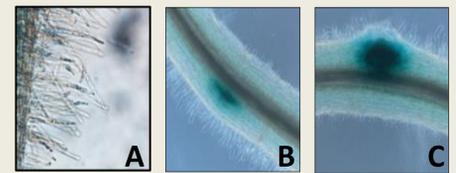


Fig. 3 - Localization of *MtN5p::GUS* transgenic roots GUS activity is visible in the root hairs at 3 hpi (A), in the root cortex at 24 hpi (B) and in nodule primordia (C) (Pii *et al.* 2012).

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.

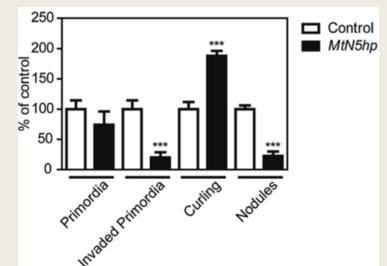


Fig. 4 – Number of root hair curling events, total and invaded primordia and mature nodules in *MtN5hp* roots inoculated with *S. meliloti*. The data reported are means \pm SE ($n=40$) calculated as percentage relatively to control inoculated roots. Student's *t* test was applied. *** $P < 0.001$ (Pii *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* develop 34% more nodules with respect to control ones, thus confirming the important role of this LTP in the establishment of the symbiosis.

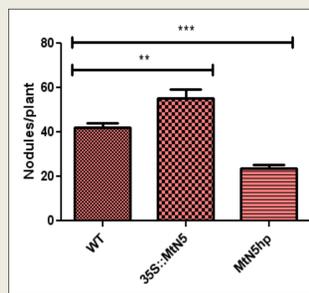


Fig. 5 – Number of mature nodules in *MtN5hp*, 35S::*MtN5* transgenic and WT plants inoculated with *S. meliloti*. The values reported are means \pm standard error (** $P < 0.01$; *** $P < 0.001$)

Medtr3g055250 and *Medtr7g052640* expression pattern during nodule development

MtN5 VQICNIDPNDLKQSCSKFVTGRNPPRADEACCGVLRANLPCLCGYKSC--ALTYYGINAKKALALPGQCGLQTPSNC--- 75
Medtr3g055250 IIVCSIDTN-KLDVCHDAITGKRPPKPTTKCCALIKKADLSCLCRYKSC--LLPALGINPTKALALPKKCGRKTTPPGCRAN 77
Medtr7g052640 MSLGNMDED-GLDACKPSVTQYPYPAKPSTCCCKALTGADLQCLCSYKNSAELPLLGIDPTLAASLPKCCDLTPPSNC--- 76
 : : * : : : : : * : * : * : : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *

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.

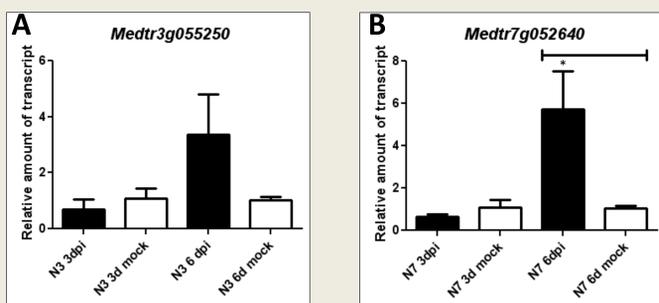


Fig. 7 – Expression level of *Medtr3g055250* (A) and *Medtr7g052640* (B) genes in *M. truncatula* 3 and 6 dpi in plants inoculated with *S. meliloti* and mock-inoculated ones. The values reported are means \pm standard error (* $P < 0.05$).

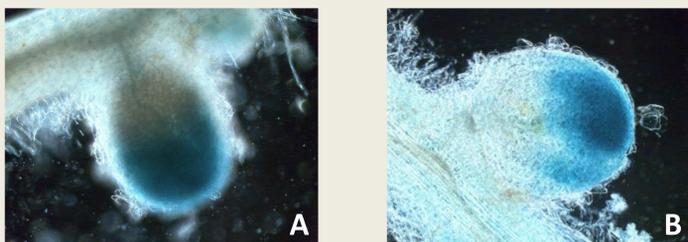


Fig. 8 – *Medtr3g055250* (A) and *Medtr7g052640* (B) promoter activity in fully developed root nodules (18 dpi).

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).

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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