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Signaling LTPs

A new plant LTPs sub-family?

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Numerous plant non specific Lipid Transfer Protein (nsLTPs) have been characterized for their antimicrobial activity, suggesting for these proteins a direct role in the protection against pathogenic microorganisms. Another group of LTPs seems to be involved in structural events in the extracellular matrix through binding and transport of hydrophobic molecules. More recently, some LTPs putatively involved in the symbiotic interaction between legumes and rhizobia have been identified. We investigated the role of *MtN5*, a LTP from *Medicago truncatula,* which is specifically expressed in the roots and induced by both *Sinorhizobium meliloti* and a root pathogenic fungus. Once the symbiosis has been established, MtN5 is preferentially accumulated in the root nodule. The suppression of *MtN5* transcript, obtained by means of an RNAi approach, resulted in a reduced nodulation, whereas its overexpression led to an increased number of nodules produced by *S. meliloti*. These observations demonstrated that MtN5 is required for an efficient nodulation. On the basis of the amino acid sequence, MtN5 has been included in the nsLTP-like sub-family, together with *Arabidopsis thaliana* DIR1, a protein playing a role in SAR signaling. The putative role(s) for this LTP in the symbiotic association are discussed in the present commentary. MtN5, together with DIR1 and other new LTPs, are proposed to form a new LTP subfamily involved in lipid signalling.

Key words: lipid transfer protein, *Medicago truncatula*, MtN5, signalling, symbiosis, *Sinorhizobium meliloti*

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Lipid transfer proteins (LTPs) are a group of cystine-rich proteins named after their ability to bind and facilitate lipid transfer between membranes in vitro.[1] Plant LTPs are usually small, basic proteins that contain a N-terminal signal peptide which targets the mature protein towards the secretory pathway.[2] For this reason, plant LTP are not believed to play a role in the intracellular trafficking of lipids and their function remains elusive. Several studies have demonstrated that plant LTPs possess antimicrobial activity in vitro and are induced during pathogen infection, suggesting that these proteins can act in the protection against pathogens.[3] LTPs seem to be implicated in the symbiotic association between rhizobia and leguminous plants as well.[4] Some LPTs identified in legume plants are induced by rhizobia infection and localized in the root nodule, the organ that hosts the symbiotic N-fixing rhizobia.[5] In legumes/rhizobia association, the host plant controls the interaction with the rhizobacteria in a quite complex way, on one hand reprogramming for compatibility and suppressing defence against symbionts, and on the other hand controlling the nodule number by limiting symbiont invasion.[6] A straightforward explanation of LTP upregulation during rhizobia infection is that these proteins could participate in the control of bacteria infection and in the autoinhibitory regulation of nodule number by the host plant. In a recent work, we have addressed the role of a root specific LTP of *Medicago truncatula* (MtN5) in the symbiotic process.[7] MtN5 was classified as an early nodulin because it is induced during the early phases of *M. truncatula/Sinorhizobium meliloti* interaction and was annotated as

Table 1. Comparison between MtN5 and the four predicted protein from *G. max*

								Pairwaise alignment with MtN5	
mRNA clone Acc.N.	mRNA length	Protein Acc.N.		Residues	Signal peptide	MW mature protein	pI	Identity	Similarity
BT091790	657	ACU16019.1	Gm1	101	26	7941	8.7	63%	85%
BT089421	625	ACU13501.1	Gm2	103	28	7908	8.7	62%	84%
BT091938	579	ACU16166	Gm3	101	26	7927	8.7	62%	84%
BT092404	596	ACU16775.1	Gm4	101	26	7841	8.7	63%	85%
Y15371	643	CAA75593	MtN5	102	27	8104	8.8	-	-

The pairwise alignments were carried out with ClustalW.[17] The signal peptides were predicted by means of SignalP3.0 software.[18] The protein Mass Weigh and pI were calculated with ProtParam tool available on Expasy Proteomic Server.[19]

a putative LTP.[5] We have demonstrated that MtN5 possesses biochemical features typical of LTPs in that it is able to bind lipids, displays inhibitory activity against microbes and it is upregulated upon root fungal infection as well as after *S. meliloti* inoculation. To study the function of MtN5 in the symbiosis between *S. meliloti* and *M. truncatula,* we silenced and overexpressed *MtN5* in roots via *Agrobacterium rhizogenes*-mediated transformation. MtN5-silenced roots inoculated by rhizobium displayed a reduced ability to nodulate: nodule number was diminished by 50–80% as compared with inoculated control roots. On the other hand, the number of nodules formed by MtN5 overexpressing roots was 3 times higher than in control roots. These results ruled out the hypothesis that MtN5 has a role in the limitation of rhizobium spreading and root colonization, suggesting that MtN5 acts rather as a positive signal in rhizobia-legumes association.

Is MtN5 a Member of a New LTP Sub-Family Involved in Lipid Signalling?

MtN5 encodes for a 643-nucleotides-long mRNA which is putatively translated in a 105 amino acids-long protein carrying a 27 residues-long signal peptide. A phylogenetic analysis between the mature sequence of the putative MtN5 protein and representative members of LTP1 and LTP2 family shows that MtN5 grouped independently with respect to the two major LTP1 and LTP2 families, whereas is closely related to the *Arabidopsis thaliana* DIR1 protein.[7] DIR1 plays a role in systemic signalling to pathogens and has been annotated as a new type of LTP.[8,9]

The amino acid sequence of MtN5 together with those of *Phaseolus vulgaris* PVR3, *Antirrhinum majus* FIL1 and *Lilium longiflorum* LIM3 have been recently used to describe a peculiar subgroup of lipid transfer proteins, known as non specific lipid transfer protein-like (nsLTP-like). The multiple sequence alignment between MtN5, and representative members of nsLTP-like group, i.e., PVR3, FIL1 and LIM3 shows that the homology is mostly restricted to the eight-cysteine motif and a few other residues (13% identity; 32% similarity), suggesting that nsLTP-like subfamily might represent a rather heterogeneous protein cluster. This observation is also supported by the fact that these proteins are implicated in a plethora of different functions, albeit all linked to developmental processes. In fact, MtN5 plays a role in the establishment of the symbiosis between *M. truncatula* and *S. meliloti*,[7] PVR3 is involved in the development of cortical cells in the roots of *P. vulgaris*,[10] FIL1 is thought to be important in petal and stamen formation[11] and LIM3 is induced during the early prophase stage of meiosis in lily microsporocytes.[12]

Recently, new full-length mRNAs (BT091790, BT089421, BT091938, BT092504) obtained from the legume soybean (*Glycine max*) (hereafter referred to as Gm1, Gm2, Gm3 and Gm4, respectively, see **Table 1**) were preliminary annotated as LTP-like proteins, on the basis of the eight-cysteine motif. A close relationship between *G. max* putative LTPs and MtN5 was highlighted by a pairwise alignment; MtN5 showed a high degree of both identity (above 60%) and similarity (above 80%) with each one of the four putative protein from *G. max* (**Table 1**). The phylogenetic relationship between well characterized members of LTP families 1 and 2, the proteins belonging to the nsLTP-like subfamily and the newly discovered Gm1, Gm2, Gm3 and Gm4 putative proteins is reported in **Figure 1A**. The phylogenetic tree obtained shows that MtN5 localizes in a sub-group of ns-LTP like proteins that contain PVR3, DIR1 and *G. max* putative LTPs, the latter being the most closely related to MtN5. It would be interesting to find out whether these soybean LTPs are induced by rhizobia and display an expression pattern similar to MtN5.

Symbiosis and interaction with pathogens are different processes that share the necessity of recognising the presence of the microorganism and activate molecular responses to it. While the response to pathogen involves defence processes, the symbiotic response requires a permissive recognition and the activation of the cascade of signals that produce the symbiotic organs. The characterization of MtN5 has pointed out the complex role played by this protein in the nodulation process during the different stages of symbiosis, in particular, in the invasion of host roots by rhizobia, in the control of pathogen attack and possibly in the formation of the mature symbiosome. A very interesting aspect is that *MtN5* is precociously induced after rhizobia inoculation (**Fig. 1B**). Gamas et al. 1996 reported that *MtN5* mRNA level increases after Nod factor treatment although in the early pre-infection phases *MtN5* induction is apparently independent from Nod factors, since *MtN5* mRNA level is transiently increased following inoculation with *nodA*- rhizobia.[13] These findings open a quite intriguing scenario, in which *MtN5* could respond to other signals, yet to be identified. Also the fact that MtN5

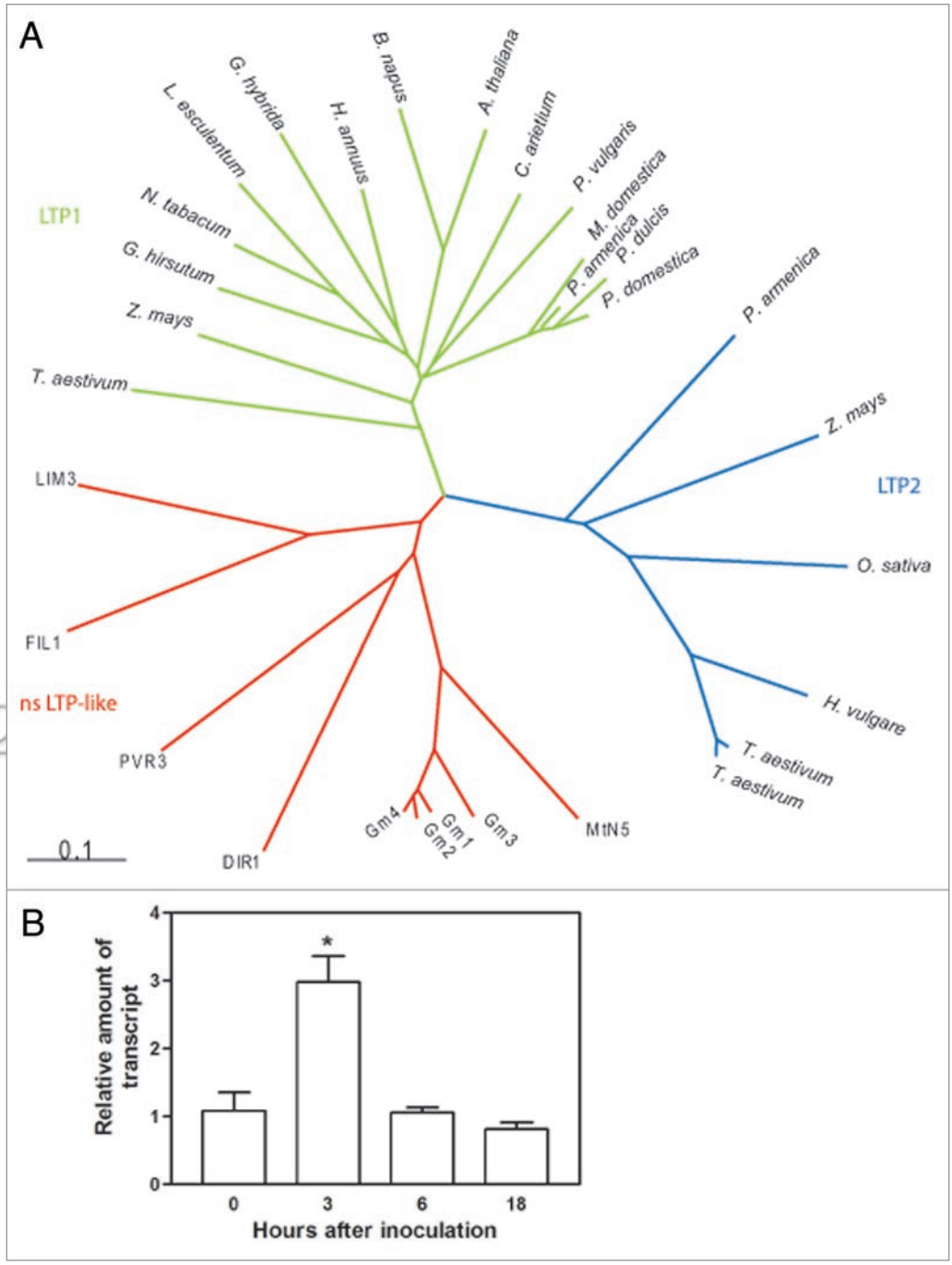

Figure 1. Phylogenetic and expression analysis of MtN5. (A) Phylogenetic tree. The sequences of the proteins were obtained from SWISS-PROT and the phylogentic tree was built with the Clustal W.[17] The protein sequences used in the phylogenetic analysis are listed as follows. For LTP1: *Cicer arietinum* (O23758), *Phaseolus vulgaris* (O24440), *Prunus dulcis* (Q43017), *Prunus domestica* (P82534), *Prunus armeniaca* (P81651), *Malus domestica* (Q9M5X7), *Nicotiana tabacum* (Q42952), *Lycopersicon esculentum* (P93224), *Gossypium hirsutum* (Q9FVA5), *Gerbera hybrida* (Q39794), *Helianthus annuus* (Q39950), *Brassica napus* (Q42614), *Arabidopsis thaliana* (Q42589), *Zea mays* (P19656), *Triticum aestivum* (Q8GZB0). For LTP2: *Triticum aestivum* (P82900 and P82901), *Hordeum vulgare* (P20145), *Oryza sativa* (P83210), *Prunus armeniaca* (P82353) and *Zea mays* (P83506). For nsLTP-like protein, sequences were obtained from NCBI databank: Gm1 (ACU16019.1), Gm2 (ACU13501.1), Gm3 (ACU16166.1), Gm4 (ACU16775.1), DIR1 (Q8W453), PVR3 (AAC49370), LIM3 (Q40227) and FIL1 (CAA40553). (B) *MtN5* expression analysis in *M. truncatula* plants. The expression on *MtN5* in the root tissue of *M. truncatula* plants was evaluated by means of quantitative RT-PCR as previously described.[7] Plants were microflood inoculated with a *S. meliloti* suspension and the sample were collected 3, 6 and 18 hours after the inoculation. The expression levels were normalized using actin as endogenous control gene. The relative expression ratios were calculated using non-inoculated root as calibrator sample. The values reported are means ± SE (n = 3). Statistical analysis were conducted using a Student's t-test. *$p < 0.05$.

accumulate in the roots after fungal infection suggests that MtN5 might participate in different signal transduction pathways controlled by different signal molecules.[7]

The phylogenetic tree indicated that MtN5, together with DIR1, clusters in a separate branch that also include new soybean putative nsLTPs. DIR1 was characterized as a long distance signal molecule, but the actual mechanism of action has still to be clarified. On the basis of these observations we might hypothesize that within the nsLTP-like subfamily, this protein cluster could have undergone a specialization toward signals transduction. The identification of a new group, the signalling LTPs, would be based not only on the sequence similarities but specially on the common characteristic of being directly involved in transduction of specific signals, either intrinsic or extrinsic.

Developmental changes requires a complex set of signals and lipid-derived molecules are now investigated as important metabolites. As an example, it has been reported that lyso-phosphatidylcholine (LPC) is directly involved in the signalling pathway of the arbuscular mycorrhizal symbiosis.[14] There are also evidence for lipid signalling to be involved in rhizobia symbiosis. It was observed that the pharmacological inhibition of phospholipase D activity prevents the expression of the early noduline *ENOD11,* unraveling a central role for lipids in the Nod factor signaling pathway.[15] Furthermore, recent findings highlighted that proteins belonging to the annexins family might also be involved in the Nod factors signaling. Annexins are ubiquitous proteins able to bind phospholipids in both Ca^{2+}-dependent and Ca^{2+}-independent manner. In plants, the majority of annexin proteins are found in the cytosol, but some can be found associated with the plasma membrane, endomembranes or the nuclear envelope. During the early stages of symbiosis, annexins are proposed either to bind membrane-derived phospholipids produced by the phospholipase activity or to act as an additional calcium channel at the nuclear envelope, participating in the generation of the nuclear associated calcium spiking.[16]

Different LTPs have to be present to recognize specific signals in different tissues. For this reason, an heterogeneous subclass of LTPs can fit in this crucial control step of plant developmental choices. Lipid derived molecules that can be recognised by LTPs, also different from typical lysolipids (i.e., lyso-phosphatidylcholine), have to be investigated in the future to clarify the signal transduction pathway mediated by the members of this new class of LTPs.

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