STUDY OF TEMPORAL AND SPATIAL EXPRESSION OF PURINE PERMEASE *PUP1* IN *MEDICAGO TRUNCATULA* USING *IN SITU* RNA-RNA HYBRIDIZATION

STUDIUL EXPRESIEI TEMPORALE SI SPAŢIALE A PURIN PERMEAZEI *PUP1* IN *MEDICAGO TRUNCATULA*, UTILIZÂND HIBRIDIZAREA *IN SITU* ARN-ARN

***KALLONIATI Chrysanthi*[1], *EFROSE Rodica-Cătălina*[2], *SKLIROS D.*[1], *KOMAITIS F.*[1], *FLEMETAKIS E.*[1*]**

[*]Corresponding author e-mail: mflem@aua.gr

Abstract. *Molecular nitrogen (N_2) is the most abundant element in the atmosphere, but not in a form that can be directly assimilated by the plants. Thus, nitrogen is a significant limiting factor for plant development and productivity. The symbiotic interaction between legumes and rhizobia, resulting to the reduction of the atmospheric nitrogen into ammonia, has been considered one of the linchpins of sustainable agriculture for many centuries. There is substantial evidence, for example, data bioinformatics analysis and functional genomics that in certain types of symbiotic nitrogen fixation, rhizobia need nucleotide bases, particularly purines. The study of the role of purines and their transport system from the host plant in rhizobium will result in a better understanding of the molecular and biochemical mechanisms underlining symbiotic nitrogen fixation and improve our ability for the genetic manipulation of this beneficial interaction. In the present study, an in situ RNA-RNA hybridization approach was performed in order to determine the temporal and spatial expression of purine permease Pup1 gene during the different stages of Medicago truncatula nodule development. In situ RNA-RNA hybridization revealed a strong expression of the purine permease Pup1 gene during nodule development. Specifically, purine permease Pup1 gene transcripts shared a similar spatial expression pattern in nodules at all developmental stages examined and were localized in the central tissue, the vascular bundles and the nodule parenchyma in nodules at 14, 21 and 28 days post inoculation.*

Key words: *Medicago truncatula,* symbiotic nitrogen fixation, purine permease, purine transport, *in situ* RNA-RNA hybridization

Rezumat. *Azotul molecular (N_2) este cel mai abundent element din natură, dar nu într-o formă care să poată fi asimilată direct de către plante. Astfel, azotul, este un factor limitativ semnificativ pentru*

[1] Department of Biotechnology, Agricultural University of Athens, Greece
[2] Department of Experimental and Applied Biology, NIRDBS-Institute of Biological Research Iasi, Romania

dezvoltarea şi productivitatea plantelor. Interacţiunea simbiotică dintre leguminoase şi bacteriile din genul Rhizobium, care are ca rezultat reducerea azotului atmosferic în amoniac, a fost considerată, de mai multe secole, una dintre bazele agriculturii durabile. Există dovezi substanţiale, de exemplu, analiza datelor bioinformatice şi de genomică funcţională care arată că, în anumite tipuri de fixare a azotului simbiotic, bacteriile au nevoie de baze azotate, în special purine. Studiul rolului purinelor şi al sistemului lor de transport din planta gazdă in bacterie, va avea ca rezultat o mai bună înţelegere a mecanismelor moleculare şi biochimice care stau la baza fixării simbiotice a azotului şi va îmbunătăţi capacitatea de manipulare genetică a acestei interacţiuni benefice. În studiul de faţă, metoda de hibridizare in situ ARN-ARN a fost utilizată pentru a determina expresia temporală şi spaţială a genei purin permeaza Pup1, în timpul diferitelor stadii de dezvoltare a nodozităţilor de Medicago truncatula. Hibridizarea in situ ARN-ARN, a relevat o expresie ridicată a genei purin permeaza Pup1 în timpul dezvoltării nodozităţilor. Mai exact, transcriptele genei Pup1 au arătat un profil similar de expresie spaţială in nodozităţi, în toate etapele de dezvoltare examinate, fiind localizate în ţesutul central, fasciculele conducătoare şi parenchimul nodozităţilor la 14, 21 şi 28 de zile după inoculare.

Cuvinte cheie: *Medicago truncatula*, fixarea simbiotică a azotului, purin permeaza, transportul purinelor, hibridizarea *in situ* ARN-ARN

INTRODUCTION

The interaction between legumes and rhizobia leads to the formation of root nodules, in which symbiotic nitrogen fixation takes place (Vance *et al*., 1997; Udvardi and Poole, 2013). Nodules are tumor-like structures that typically possess three structurally and physiologically distinct zones called external cortex, internal cortex, and central region. Symbiotic cells are localized in the central region and are interspersed with non-symbiotic cells. Bacteroids (the symbiotic form of rhizobacteria) are confined inside cytoplasmic vesicles (symbiosomes) of plasmalemma origin, in symbiotic cells and can convert biochemically the molecular nitrogen to ammonia. This symbiosis is beneficial for both symbionts, since the needs of the plant for nitrogen are largely covered by rhizobia and the plant, in turn, provides rhizobia with organic carbon produced during photosynthesis (Udvardi, 1997).

Purine nucleotides are essential precursors for nucleic acids (DNA and RNA) and are among the most important nitrogen compounds in all living organisms (Verlag *et al*, 2003). Besides, purines are components of many coenzymes involved in reactions such as energy transfer, transfer of organic molecules and redox reactions. Purines are involved in vital cellular processes in all organisms since they can be used as nitrogen or carbon sources. Moreover, adenosine triphosphate (ATP), which contains the purine adenine, is a "molecular unit of currency" of intracellular energy transfer in all biological systems, and nucleotide derivatives are involved in biosynthetic processes, such as UDP-

glucose to glycogen synthesis. Cyclic nucleotides such as cAMP and cGMP are 'messengers' of intracellular and intercellular signals (Berg and Jørgensen, 2006).

The fact that the symbiotic system of *Medicago truncatula - Sinorhizobium meliloti* forms indeterminate nodules, in which bacteroids continuously undergo endoreduplication (successive genome amplification cycles without subsequent cell division) (Terpolilli *et al.*, 2012) creates a need for continuous supply of purines in nodules. In order to gain an insight into the biochemical and physiological role of purine transport and metabolism in nodules during symbiotic nitrogen fixation, we studied the temporal and spatial expression of purine permease *Pup1* in *M. truncatula* nodules at various developmental stages using *in situ* RNA-RNA hybridization.

MATERIAL AND METHOD

The *S. meliloti* strain 1021 (Leong *et al*., 1985) was typically grown at 28°C on mannitol-rich medium supplemented with tetracycline at 5 µg/ml. Prior to germination, *M. truncatula* seeds were scarified for 5 min with H_2SO_4, sterilized for 20 min in 2% (v/v) NaOCl-0.02% (v/v) Tween 20, pregerminated at 18°C in the dark for 72 h, and spot inoculated with a suspension culture of the rhizobial strain at an OD_{600} of 0.1. Plants were grown in a controlled environment with a 16h day/8h night cycle at 22 °C and at 132W light intensity and watered with a low-nitrogen B&D solution alternately with dH_20 every two days.

In situ hybridization experiments were performed as previously described (Scheres *et al*., 1990; Flemetakis *et al*., 2000). *M. truncatula* nodules harvested at 14, 21 and 28 days post inoculation with *S. meliloti*, were fixed in 4% (w/v) paraformaldehyde supplemented with 0.25% (v/v) glutaraldehyde in 10 mM sodium phosphate buffer (pH 7.4) for 4 h in a vacuum aspirator. Fixed nodules were block-stained in 0.5% (w/v) safranin, dehydrated through ethanol series, embedded in paraffin and 7 µm-thin sections were cut. Antisense and sense RNA probes labeled with digoxigenin-11-rUTP (Boehringer Mannheim, Mannheim, Germany) were transcribed from the cDNA clone encoding for *Pup1* gene (Gene ID Medtr2g015470.1). The probe was designed close to the 3'-UTR of the gene and its length was 150 bp. Sections were prepared for hybridization as described before (Scheres *et al*., 1990) and hybridized overnight at 42 °C in 50% (v/v) formamide, 300 mM NaCl, 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.025% (w/v) bovine serum albumin (BSA), 10% (v/v) dextran sulfate and 60 mM DTT. After hybridization, the sections were treated with a solution containing 500 mM NaCl, 1mM EDTA, 10 mM Tris HCl and 50 lg/ml RNase A. Finally, sections were washed several times in a 2xSSC solution. Hybridization signals were visualized with anti-digoxigenin antibodies conjugated with alkaline phosphatase. Images were processed and prepared for presentation with CorelDraw X4 software.

RESULTS AND DISCUSSIONS

For the spatial and temporal localization of purine permease *Pup1* gene transcripts in *M. truncatula* nodules was studied using *in situ* RNA-RNA hybridization. For this purpose, *M. truncatula* nodules were carefully harvested at 14, 21 and 28 days post inoculation with *S. meliloti* and baptized in fixation buffer. The nodules were dehydrated and embedded in paraffin. Then, 7µm thin

sections were obtained and transferred to properly treated slides. Section hybridization was performed with labeled antisense RNA, which was obtained by *in vitro* transcription of the cDNA clone encoding for purine permease, *Pup1*, using appropriate RNA polymerase. RNA labeling was performed using digoxigenin-labeled uridino-triphosphate nucleotides (DIG-11-rUTP).

The hybridization signal was detected using anti-DIG antibodies carrying the alkaline phosphatase enzyme. Alkaline phosphatase catalyzes the formation of chromophore precipitate, in the presence of the NBT/BCIP substrate. The hybridization signal appears as a blue-purple precipitate. Sections were used as controls which were hybridized to sense RNA.

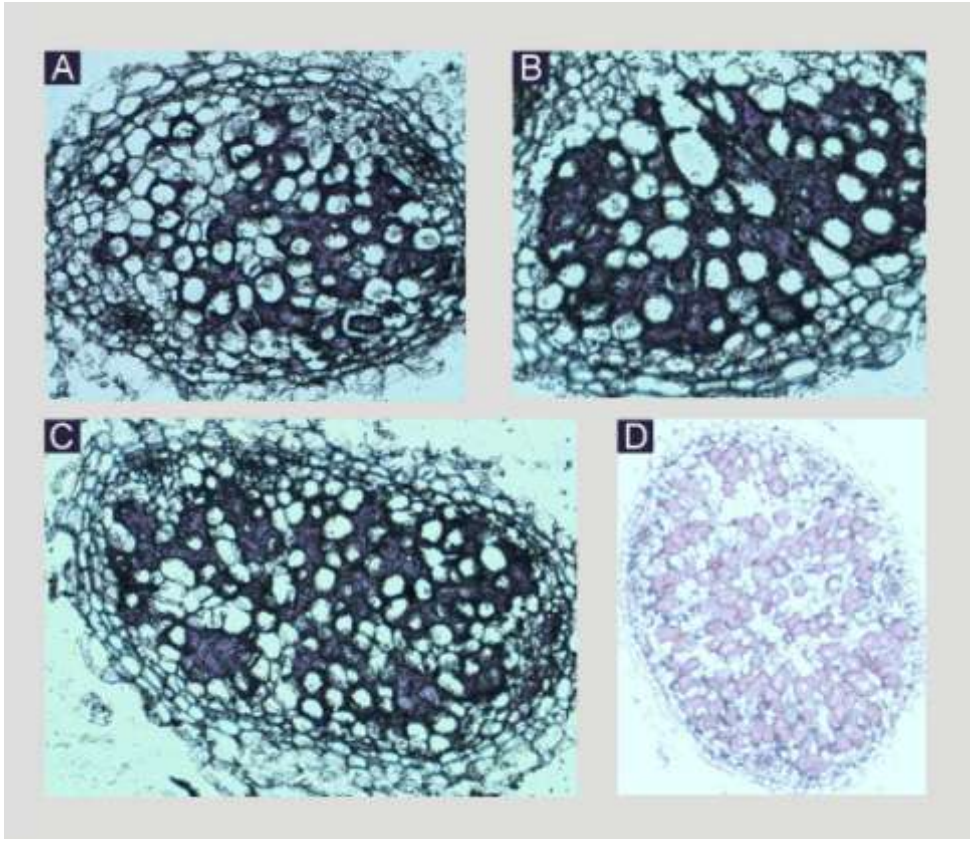

Fig. 1 *In situ* temporal and spatial localization of purine permease *Pup1* gene transcripts in developing *M. truncatula* nodules. *In situ* RNA-RNA hybridization for the detection of purine permease *Pup1* transcripts in *M. truncatula* nodules at various developmental stages. Hybridization signal is visible as blue-purple precipitate. (A, B, C) Nodule sections at 14, 21 and 28 days post inoculation with *S. meliloti*, respectively were hybridized with *Pup1* antisense DIG-11-rUTP labeled RNA probe. (D) Nodule section was hybridized with *Pup1* sense DIG-11-rUTP labeled RNA probe.

In young nodules, harvested at 14 days post inoculation, the hybridization signal of purine permease *Pup1* transcripts, appears evenly distributed in all

tissues of the developing nodule, i.e. in the central tissue, the vascular bundles and the nodule parenchyma (fig. 1A). In nitrogen-fixing nodules at 21 days post inoculation, the hybridization signal is observed again in all nodule tissues (fig. 1B). Finally, in mature nodules 28 days post inoculation, the hybridization signal appears evenly distributed in the nodule parenchyma, the vascular bundles and the central tissue of the nodule (fig. 1C). In all of the above hybridizations, sections of nodules were used as a control, in which sense RNA was transcribed from the corresponding purine permease *Pup1* clone, (Fig. 1D). In the case of sense RNA, no specific signal appeared in any group of cells, confirming that the signal shown in the above cases was due only to the presence of the mRNAs of the purine permease *Pup1* gene.

Fedorova *et al*., 2002 has reported high nodule-specific expression of purine permease. The strong hybridization signal of purine permease *Pup1* transcripts observed in all developmental stages of the nodules indicates an important role of purine permease *Pup1* in nodules, during symbiotic nitrogen fixation, which was previously overlooked. Furthermore, the biochemical characterization of purine transporters through heterologous expression systems will be performed and the physiological role of *M. truncatula* and *S. meliloti* genes involved in purine transport and metabolism during symbiotic nitrogen fixation will be analysed in order to study the biochemical and physiological role of purine transport and metabolism in nodules during symbiotic nitrogen fixation and the possible exchange of purines between the two symbionts, an aspect that has not been investigated at all in the past.

CONCLUSIONS

1. Hybridization with sense RNA confirmed that the signal observed in the nodules at various developmental stages was due only to the presence of the mRNAs of the purine permease *Pup1* gene.

2. *In situ* RNA-RNA hybridization approach revealed a strong expression of the purine permease *Pup1* gene during nodule development. Furthermore, purine permease *Pup1* gene transcripts are localized in the central tissue, the vascular bundles and the nodule parenchyma in young nodules at 14 days post inoculation. In mature nodules, at 21 and 28 days post inoculation, purine permease *Pup1* gene shared a similar spatial expression pattern.

3. The biochemical characterization of purine transporters through heterologous expression systems and the analysis of the physiological role of *M. truncatula* and *S. meliloti* genes involved in purine transport and metabolism during symbiotic nitrogen fixation will allow to investigate the biochemical and physiological role of purine transport and metabolism in nodules during symbiotic nitrogen fixation and the possible exchange of purines between the two symbionts, an aspect that has not been investigated at all in the past.

***Acknowledgments**: This research was supported by The State Scholarship Foundation which implements scholarship programs in natural sciences, aiming at post-doctoral research in Greece. The project is co-funded by Greece and the European Union (European Social Fund) through the Operational Program "Human Resources Development, Education and Lifelong Learning 2014-2020"*

REFERENCES

1. **Berg G.M., Jørgensen N.O.G., 2006** - *Purine and pyrimidine metabolism by estuarine bacteria*. Aquatic Microbial Ecology. 42, p. 215-226.
2. **Fedorova M., van de Mortel J., Matsumoto P.A., Cho J., Town C.D., Van den Bosch K.A., Gantt J. S. and Vance C.P., 2002** - *Genome-Wide Identification of Nodule-Specific Transcripts in the Model Legume Medicago truncatula*, Plant Physiology. 130, p. 519-537.
3. **Flemetakis E., Kavroulakis N., Quaedvlieg N.E., Spaink H.P., Dimou M., Roussis A. and Katinakis P., 2000** - *Lotus japonicus contains two distinct ENOD40 genes that are expressed in symbiotic, nonsymbiotic, and embryonic tissues*, Mol. Plant-Microbe Interact.13, p. 987–994.
4. **Leong S., Williams P. and Ditta G., 1985 -** *Analysis of the 5' regulatory region of the Gene for delta-aminolevulinic acid synthetase of Rhizobium meliloti.* Nucleic Acids Research. 13, p. 5965-5976.
5. **Scheres B., van de Wiel C., Zalensky A., Horvath B., Spaink H.P., van Eck H., Zwartkruis F., Wolters A., Gloudemans T., van Kammen A. anad Bisseling T., 1990 -** *The ENOD12 gene product is involved in the infection process during the pea-Rhizobium interaction*, Cell. 60, p. 281-294.
6. **Terpolilli J.J., Hood G.A., Poole P.S, 2012** - *What determines the efficiency of N(2)-fixing Rhizobium-legume symbioses?* Adv. Microb. Physiol. 60, p. 325-89.
7. **Udvardi D., 1997** - *Metabolite transport across symbiotic membranes of legume nodules.* Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, p. 493-523.
8. **Udvardi M., and Poole P.S., 2013** - *Transport and metabolism in legume-rhizobia symbioses.* Annu. Rev. Plant Biol. 64, p. 781–805.
9. **Vance C.P., Miller S.S., Driscol B.T., Robinson D.L., Trepp G., Gantt J.S., and Samas D.A., 1997** - *Nodule carbon metabolism: organic acids for N_2 fixation*. P. In: (Elmerich, C., Kondorosi, A., Newton, W.E., Eds.) *Biological Nitrogen Fixation for 21th Century*. Kluwer Academic Publishers, London. p. 443-448.
10. **Verlag F., Stasolla C., Katahira R., Thorpe T.A. and Ashihara H., 2003** - *Purine and pyrimidine nucleotide metabolism in higher plants*, 1295.