Putatively hypoxia-regulated genes that control the carbon allocation and metabolism in the nodule of *Medicago truncatula*

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Co-financed by Greece and the European Union

Abstract

Although N_2 is extremely abundant, comprising about 79% of the atmosphere, plants cannot convert it to useful organic forms and mineral nitrogen is limited in soils. Legumes are unique among crop plants in the ability to fix N_2 in symbiotic association with bacteria called rhizobia. Symbiotic nitrogen fixation (SNF) takes place in legume root nodules, organs of tumor-like structure, and is accomplished by the bacterial nitrogenase, an enzyme that requires hypoxic microenvironment (10 to 40 nM of O_2) and high ATP levels for its activity. Hypoxia has been shown to alter the expression of genes involved in several metabolic pathways with the exact response being plant organ-specific. Here, we have used the model symbiotic system of *Medicago truncatula* – *Sinorhizobium meliloti* to identify plant genes involved in carbon allocation and metabolism in the nodule, with their expression being putatively under hypoxia-related regulation. *M. truncatula* is an excellent candidate for such studies, due to the available databases regarding the sequencing of the genome (http://mtgea.noble.org/v2, http://www.medicago.org/genome, NCBI), the expression of genes (http://mtgea.noble.org/v2), and the active metabolic pathways (http://www.genome.jp/kegg/pathway.html), as well as, the existence of established Tnt1-insertion mutant lines. *In silico* analysis was conducted to identify *M. truncatula* genes encoding for sugar transporters and glycolysis enzymes isoforms that are nodule-specifically expressed or nodule-highly induced. A small number of such genes were identified; however all the corresponding encoded proteins control significant regulatory steps of carbon allocation and metabolism in the plant cell. To verify the *in silico* analysis, total RNA was extracted from different organs and nodule developmental stages of *M. truncatula*, and the expression of these genes (RT-qPCR) is depicted. Furthermore, we present data concerning the structure of these genes, the prediction of the tertiary structure along with the annotated functions of the encoded proteins. The corresponding cDNAs were obtained and the coded sequences of these genes were cloned. Moreover, we present data concerning the spatial expression and the subcellular localization of the products of these genes in the nodule of *M. truncatula* and results concerning their physiological role during SNF.

The authors thank the Ministry of Education, Lifelong Learning and Religious Affairs for the financial assistance provided. This work was performed within the grant program ARISTEIA II, co-funded by the European Union – European Social Fund & National Resources.

One of the authors, F. Komaitis thanks the Onassis Foundation for the scholarship granted to him.

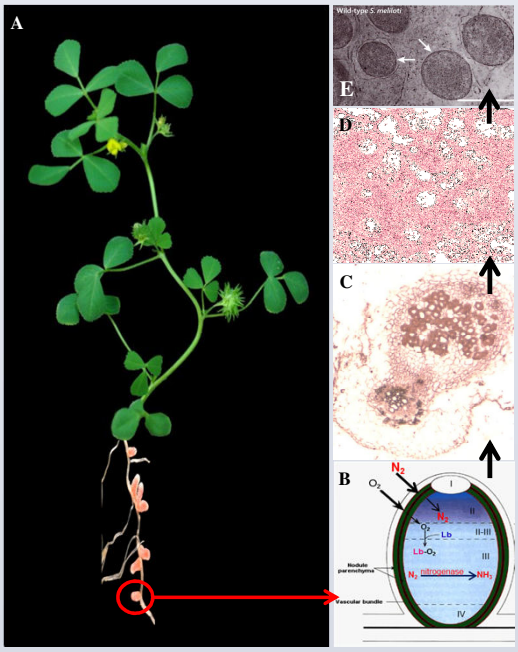


Figure 1: *M. truncatula* legume plant. A root nodule is noted (A). Nodule hypoxic microenvironment: A diffusion barrier that regulates gas flux and the expression of leghemoglobins facilitates low levels of free-oxygen to prevent the inactivation of the oxygen-sensitive bacterial nitrogenase (B). Section of a paraffin embedded nodule, nitrogen fixing symbiotic cells are located in the central nodule region (cells with expanded cytoplasm) (C). Magnification of the previous section. Symbiosomes are visible inside the nitrogen fixing symbiotic cells (D). Symbiosomes containing bacteroids of *S. meliloti* inside a nitrogen fixing symbiotic cell (E).

Materials & Methods

- *In silico* analysis in public databases: KEGG pathway database, JCVI: Medicago & NCBI databases and MtGEA (Noble Institute)
- Protein structure prediction (Phyre2, Imperial College, London)
- *M. truncatula* plant development
- qPCR technique
- *In situ* RNA-RNA hybridization

Results

Gene	Description	Gene	Description
Medtr2g100710	**6-Phosphofructokinase**	Medtr5g019870	Monosaccharide transporter
Medtr1g101950	**Fructokinase**	Medtr1g104780	Monosaccharide transporter

Table 1: Genes encoded for sugar transporters and glycolysis enzymes isoforms of *M. truncatula* found to be nodule-specifically expressed or nodule-highly induced

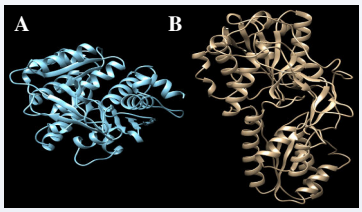
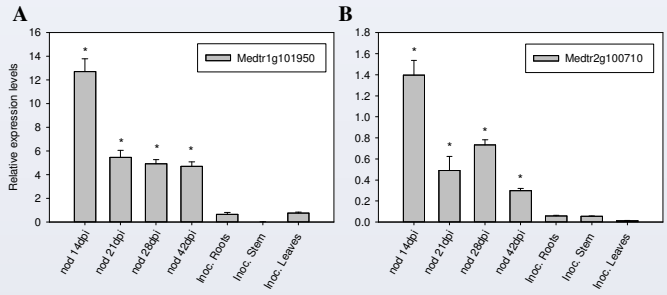


Figure 2: Tertiary structure prediction for the proteins that are coded by Medtr1g101950 (A) and Medtr2g100710 (B) genes.



Graph 1: Relative expression levels of *M. truncatula* Medtr1g101950 (A) and Medtr2g100710 (B) genes in different nodule developmental stages and in non-symbiotic organs. Bars represent means +/- SE of independent biological repeats (n=3). ANOVA test was conducted between the treatments and the asterisks note statistically significant differences in relative expression levels.

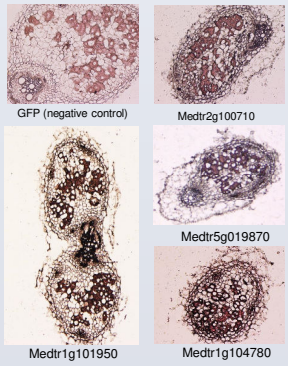


Figure 3: *In situ* RNA-RNA hybridization in nodule sections. The antisense RNA, for each gene mentioned, was used as a probe.

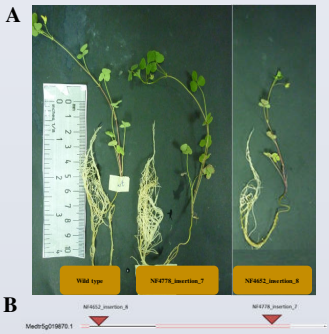
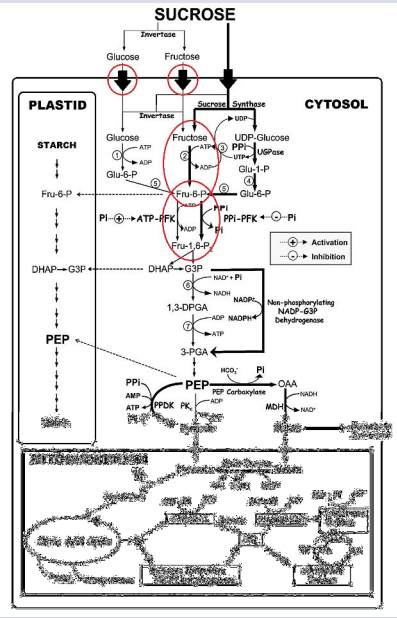


Figure 4: Phenotypical differences among wild-type *M. truncatula* and two mutant lines concerning Medtr5g019870 gene (A), Medtr5g019870 gene structure and sites of the above Tnt-1 insertions (B).

Conclusions



A. *In silico* analysis in *M. truncatula* databases and qPCR results identified four nodule specific expressed or highly induced genes, encoded for sugar transporters and glycolysis enzymes isoforms.

B. Encoded proteins regulate glycolysis pathway, as predicted with the function of fructokinase (Medtr1g101950), 6-phosphorofructokinase (Medtr2g100710) and monosaccharide transporters (Medtr1g104780, Medtr5g019870).

C. All genes found to be expressed in the nodule tissue with strong probability of being expressed inside the nitrogen fixative cells and their neighbors.

D. Mutant plants that have knock-out Medtr5g019870 gene shows a phenotype that the severity of the effect on plant growth depends of the site of the Tnt-1 insertion into the gene.

Figure 5: Plant glycolysis metabolic pathway. The sites of enzymatic activity coded by the identified genes are noted with a red circle.