# Nodule-specifically Expressed Genes involved in the Carbon allocation and metabolism in the Nodule of *Medicago truncatula*

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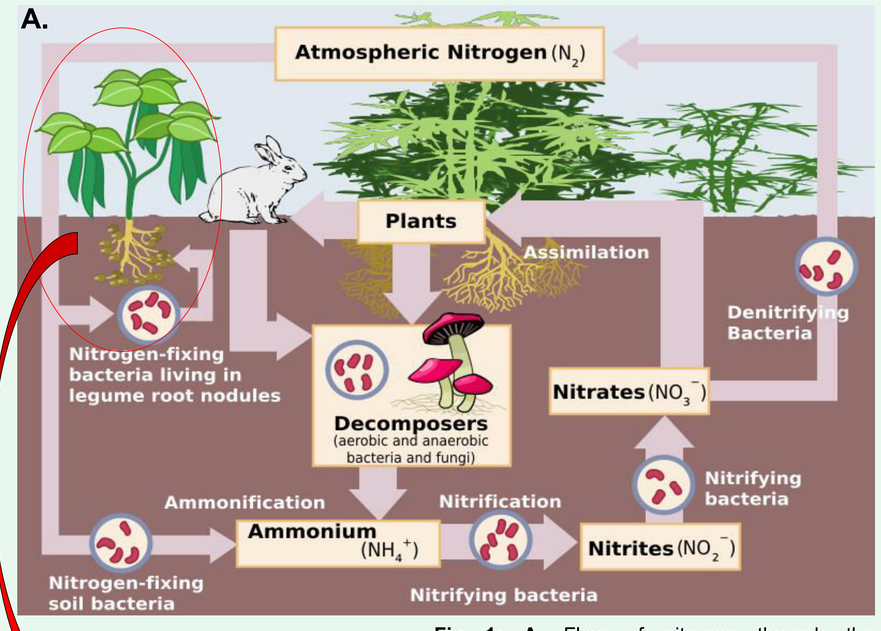
## Abstract

Although N2 is 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 their ability to fix $N_2$ in symbiotic association with rhizobia**. Symbiotic nitrogen fixation** (SNF) takes place in legume root nodules, organs of tumor-like structure, and is accomplished by nitrogenase. SNF is an energy demanding process, fueled by plant photosynthate that is transported to the nodules as sucrose. There, **sugar transport and glycolysis** facilitates the production of dicarboxylic acids, the final compounds supplied to bacterial symbionts performing SNF. 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**. *M. truncatula* is an excellent candidate for such studies, due to the available databases concerning the sequencing of the genome, the expression of genes, the active metabolic pathways, and 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 that are nodule-specifically expressed or nodule-highly induced. Few such genes were identified; however 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 is depicted. We present data concerning the structure of these genes, the amino acid sequence, and the predicted secondary structure along with annotated functions of the encoded proteins. Corresponding cDNAs were generated and the coded sequences of these genes were cloned. Finally, we present data concerning the spatial expression and subcellular localization of the gene products in the nodule of *M. truncatula* and results concerning their physiological role during SNF.



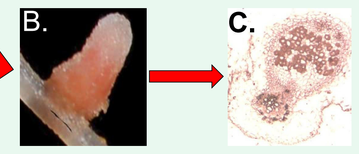




**Fig 1: A.** Flow of nitrogen through the environment. Symbiotic nitrogen fixation part is enclosed in the red circle. **B.** Nodule of *M. truncatula*. **C.** Section of a paraffin embedded nodule, nitrogen fixing symbiotic cells are located in the central nodule region (cells with expanded cytoplasm).

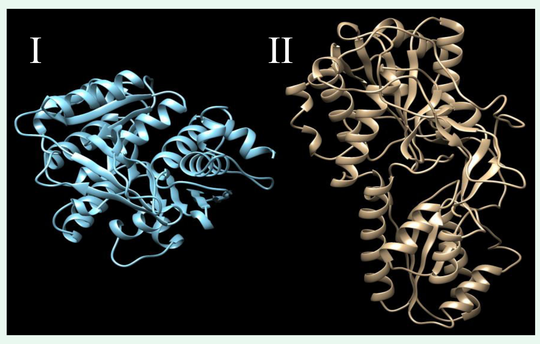
## Materials and Methods

- *In silico* analysis using Noble Foundation's *M. truncatula* Expression Atlas (MtGEA), JCVI: Medicago and KEGG pathway databases.
- Prediction of transmembrane helices in proteins (TMHMM Server v2.0).
- Protein structure prediction (Phyre2, Imperial College, London).
- Growth of *M. truncatula* plants and inoculation with *S. meliloti*.
- RNA isolation and cDNA synthesis.
- Design of gene-specific primers.
- qPCR to estimate gene expression levels.
- Cloning of gene encoded sequences.
- Identification of homozygous mutant plants.
- *In situ* RNA-RNA hybridization.

## Results

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

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



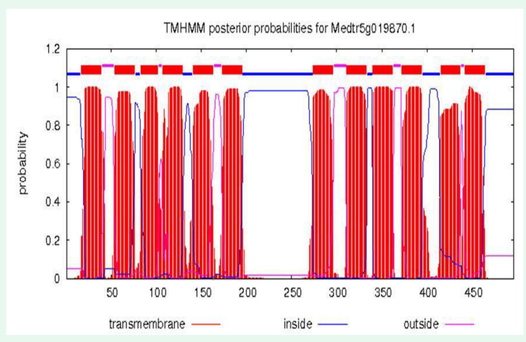




**Fig 2:** (Left) Tertiary structure prediction of the proteins that are coded by Medtr1g101950 (I) and Medtr2g100710 (II) genes. (Right) Predicted secondary structure of Medtr5g019870 and Medtr1g104780 transporters.

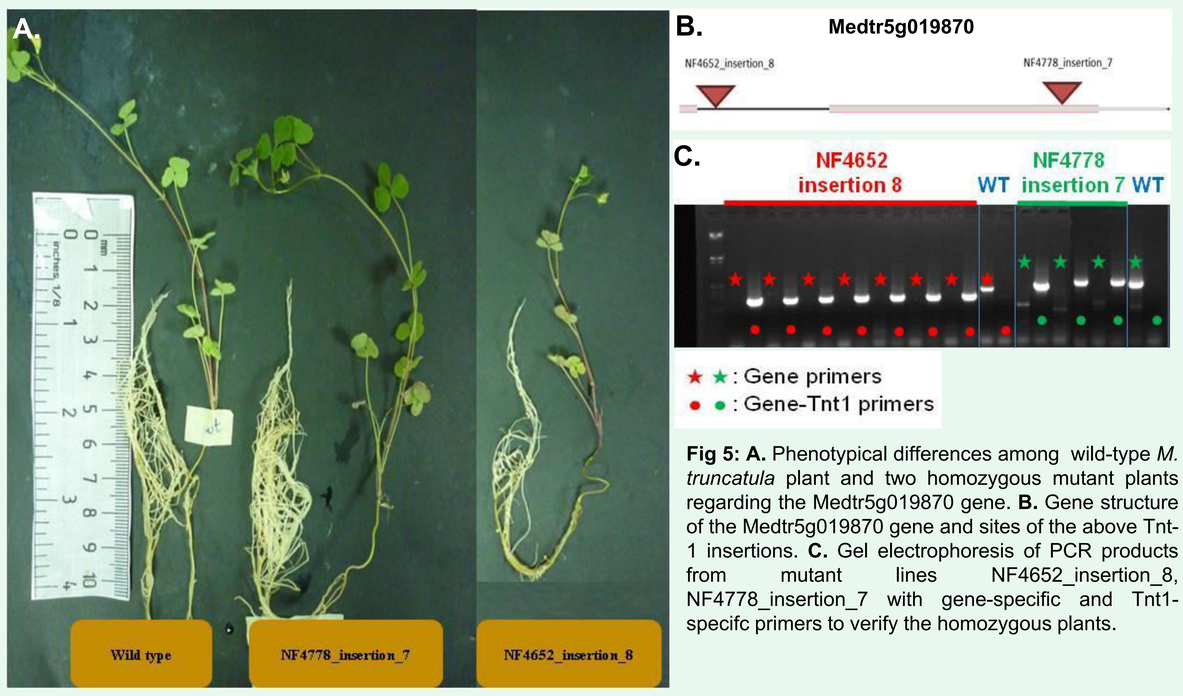



**Fig 5: A.** Phenotypical differences among wild-type *M. truncatula* plant and two homozygous mutant plants regarding the Medtr5g019870 gene. **B.** Gene structure of the Medtr5g019870 gene and sites of the above Tnt-1 insertions. **C.** Gel electrophoresis of PCR products from mutant lines NF4652_insertion_8, NF4778_insertion_7 with gene-specific and Tnt1-specifc primers to verify the homozygous plants.

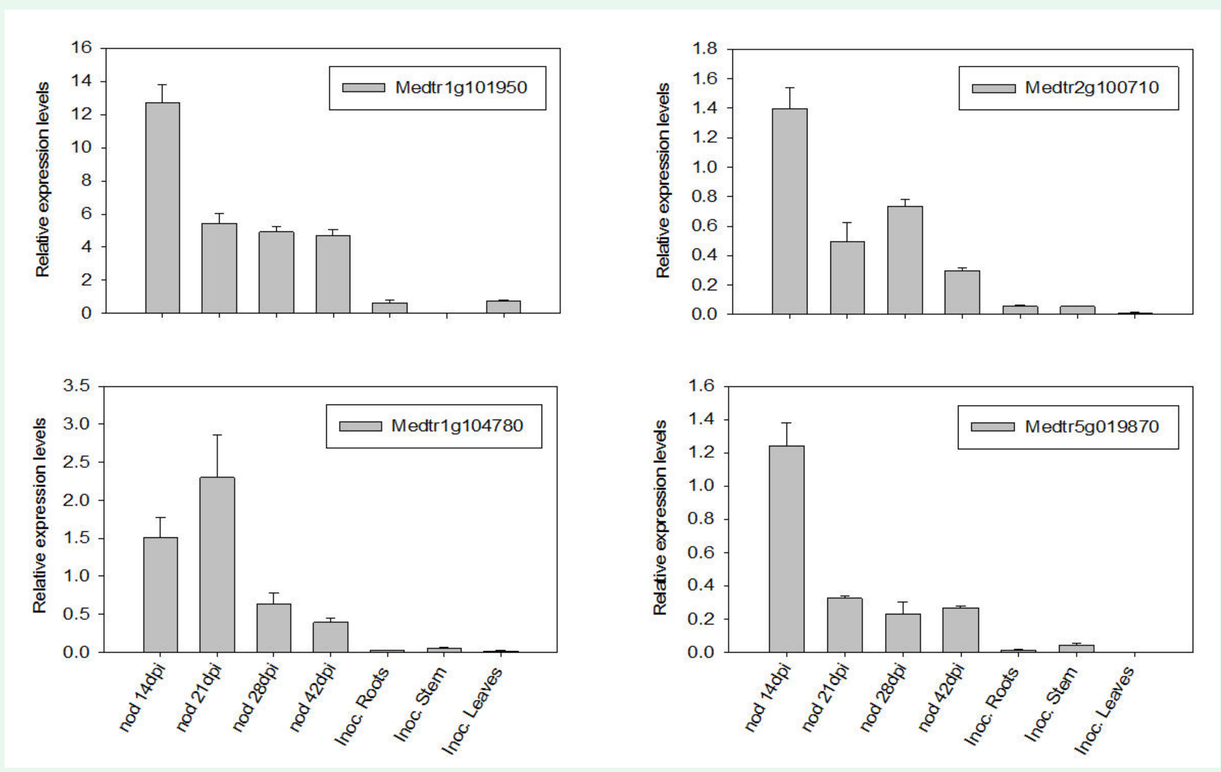



**Fig 3:** Relative expression levels of *M. Truncatula.* Medtr1g101950, Medtr2g100710, Medtr1g104780 and Medtr5g019870 genes in different nodule developmental stages and in non-symbiotic organs (the expression of Medtr5g014320 gene was used as reference). Bars represent means +/- SE of independent biological repeats (n=3). ANOVA test was conducted between the treatments.

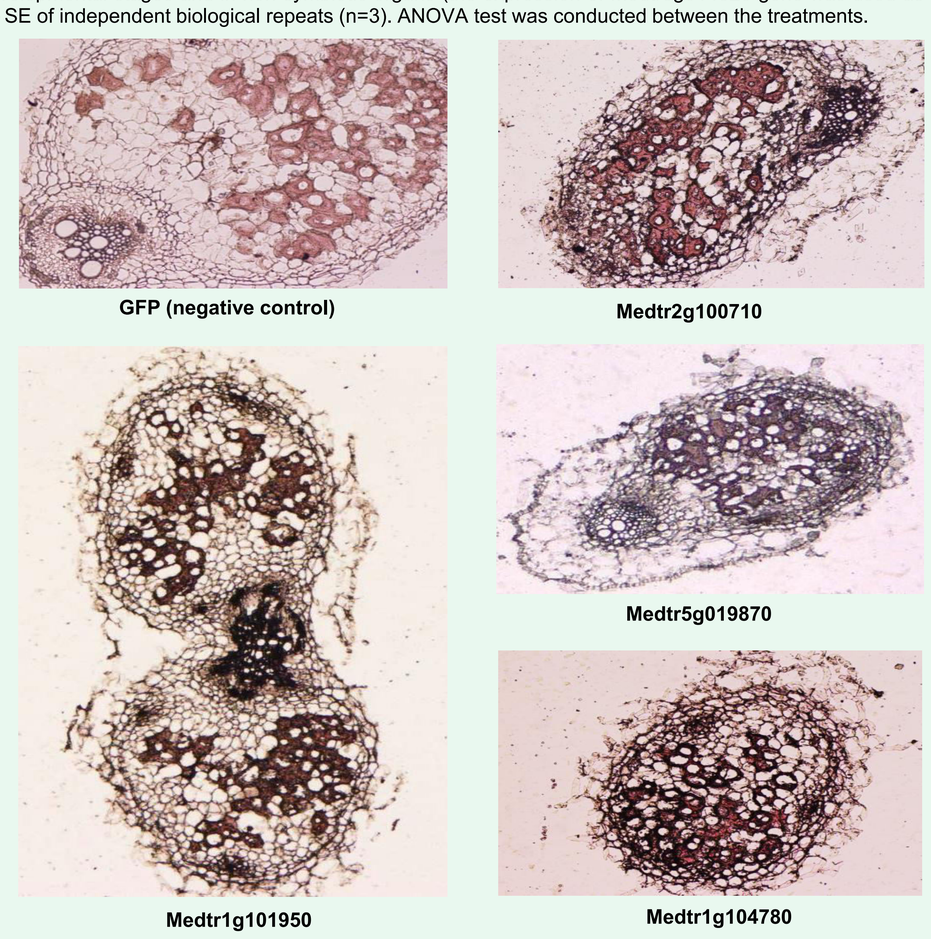



**Fig 4:** *In situ* RNA-RNA hybridization in nodule sections. The antisense RNA, for each gene mentioned, was used as a probe. Signal is found inside nitrogen fixing symbiotic cells and their surrounding cells, as well as in the nodule vascular bundle tissue cells.

## Conclusions

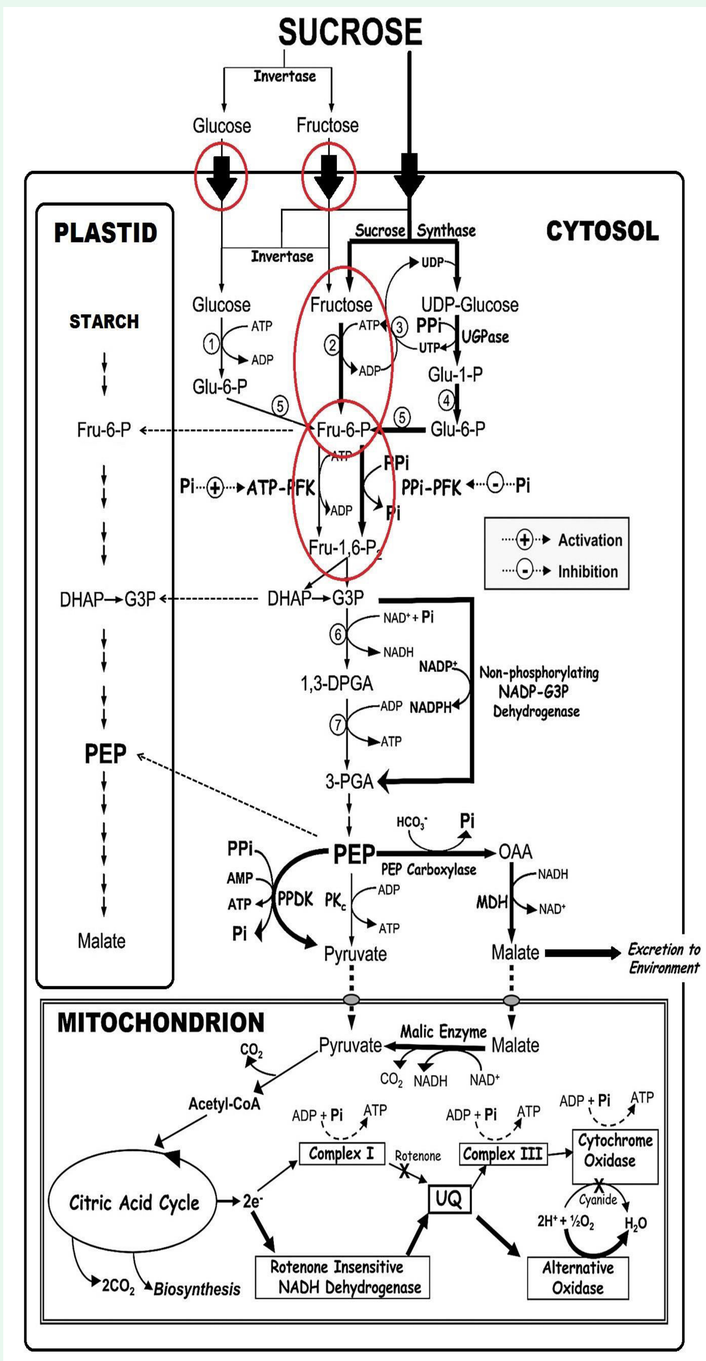



**Fig 6:** Plant glycolysis metabolic pathway. The sites of enzymatic activity coded by the identified genes are noted with a red circle.

- *In silico* analysis and qPCR results identified **4 nodule specifically-expressed or highly-induced genes**, encoding for sugar transporters and glycolysis enzymes isoforms.
- Predicted secondary structure of the encoded proteins is presented.
- Encoded proteins predicted to function as: **fructokinase** (Medtr1g101950), **6-phosphofructokinase** (Medtr2g100710), **monosaccharide transporters** (Medtr1g104780, Medtr5g019870) and possibly represent significant regulation enzymes of glycolysis pathway.
- There is a high possibility that genes found to be highly expressed in the nodule tissue are also expressed in the nitrogen fixative and their neighboring cells.
- The severity of the effect of Medrt5g019870 mutants on plant growth is dependent on the site of the Tnt-1 insertion into the gene.

Ευρωπαϊκή Ένωση
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