# THE EXPRESSION PATTERN OF TWO ALPHA – TYPE CARBONIC ANHYDRASE GENES IN DIFFERENT ORGANS OF *LOTUS JAPONICUS* L.

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**Key words:** *Lotus japonicus* L., carbonic anhydrase, reverse transcription- polymerase chain reaction (RT) – PCR.

**Abstract:** The relative abundance of cellular RNA transcripts is a commonly studied parameter of genes expression. Reverse transcription followed by the PCR (RT – PCR) leading to amplification of specific RNA sequence in cDNA form is a sensitive means for detecting RNA molecules, a means for obtaining material for sequence determination and a step in cloning a cDNA copy of the RNA. The expression pattern of two carbonic anhydrase genes (LjCA α1 and LjCA α 2 - type) was determined in different organs of *Lotus japonicus* L.

## INTRODUCTION

Carbonic anhydrase (CA, E.C. 4.2.1.1.) is a zinc-containing enzyme that catalyzes the reverse hydration of $CO_2$ to bicarbonate. It is a primitive and ubiquitous enzyme, widely distributed throughout nature, from eukaryotes such as vertebrates, invertebrates, and plants, to prokaryotes such as archaebacteria and eubacteria, found in virtually every tissue and cell type, in many subcellular organelles (Maren, 1967; Dodgson et al., 1991; Badger and Price, 1994; Smith et al. 1999; Smith and Ferry, 2000, Moroney et al., 2001).

All carbonic anhydrases are divided into three genetically distinct classes (α, β and γ) that have no significant sequence homology and evolved independently. Thus, the carbonic anhydrase classes are excellent examples of convergent evolution of catalytic function (Tashian, R.E., 1989).

The α–CAs are found primarily in animals, but homologs have also been identified in the bacterium *Neisseria gonorrhoeae* and the green-alga *Chlamydomonas reinhardtii* (Fukusawa et. al., 1990; Zhu, X., and Sly, W., 1990; Tashian, 1992; Sültemeyer, et al., 1993; Hewett-Emmet and Tashian, 1996).

CAs belonging to the β–CA class has been found in both $C_3$ and $C_4$ monocot and dicot plants, in the mitochondria of *C. reinhardtii* and in various eubacteria (Yagawa et al., 1988; Hewett-Emmet and Tashian, 1996; Smith, K.S. and J.G.Ferry, 1999).

The γ-CAs is a newly discovered gene class, with the enzyme from *Mathanosarcina thermophila* being the only γ-CA isolated and characterized thus far (Alber and Ferry, 1994; Parisi et al., 2000).

In spite of the differences in sequence, all forms of CA have an essential zinc ion in the active site. All carbonic anhydrases are completely different from one another at the level of their tertiary and quaternary structures, but the active sites show essential features of remarkable similarity (Kimber and Pai, 2000; Mitsuhashi et al., 2000; Kisker et al., 1996).

In spite of the differences highlighted above, these enzymes catalyze the same reactions, in which the zinc ion activates a water molecule that reacts with carbon dioxide, or destabilize bicarbonate in reverse reaction. Even though the residues that have essential functions are different, the catalytic mechanisms are probably very similar.

The widespread abundance of carbonic anhydrase isoforms in plants, animals and microorganisms suggests that possibly this enzyme participates in a broad range of diverge physiological and biochemical processes, including pH regulation, $CO_2$ and bicarbonate transport, ion transport and water end electrolyte balance (Henry, 1996). Metabolic roles include important steps in pyrimidine biosynthesis, gluconeogenesis and lipogenesis, as all these processes require bicarbonate for the initial carboxylation reactions.

In green plants, the only well established physiological role of CA is to provide adequate levels of inorganic carbon for carboxylases such as ribulose-biphosphate carboxilase (Rubisco).

The role of CA in dark $CO_2$ fixation is expected to be significant since it provides the substrate for the carboxylation of oxaloacetate by phosphoenolpyruvate carboxylase (Chollet et al., 1996). In symbiotic nitrogen fixation, dark $CO_2$ fixation may also play an important role. It has been suggested that the dark $CO_2$ fixation may provide a large fraction (30%) of the carbon skeletons for amide synthesis or bacteroid metabolism (Rosendal et al., 1990).

The aim of the study was to investigate the expression pattern of two carbonic anhydrases genes belongs to the α-class, in different organs of *Lotus japonicus* L..

## MATERIALS AND METHODS

**PLANT MATERIAL AND GROWTH CONDITIONS**

*Lotus japonicus* (Cultivar Gifu B-129) seeds were kindly provided by Dr. Jens Stougaard (University of Aarhus, Denmark). The plants were grown in a controlled environment with an 18-h-day/ 6h- night cycle, a 22°C day/ 18°C night regime and 70% humidity (Handberg and Stougaard, 1992). Prior to germination, seeds were soaked for 5 min with $H_2SO_4$ and then sterilized for 20 min in a solution containing 2% NaOCl- 0.02% Tween 20. Seeds were pregerminated at 18°C in the dark for 72 h and the small plants were grown with Holland nutrient solution. For the inoculation with rhizobia, 72h seedlings were inoculated with a 0.1 OD600 suspension culture of *Mesorhizobium loti* (strain E1R.pMP2112) and the plants were grown in nitrogen-free BXD nutrient solution. The day of infection was considered day 0.

**RT-PCR A NALYSIS**

To analyze carbonic anhydrase genes expression, total RNA was isolated from different *Lotus japonicus* tissues (nodules, leaves, stems, roots, flowers, green seedpods, germinated hypocotyls, germinated cotyledons and apical meristems) and quantified by spectrophotometry and agarose gel electrophoresis ( Brusslan and Tobin, 1992).

Prior to RT-PCR, the total RNA samples were treated with DNAse I (Promega, Madison, WI) at 37°C for 10 min, in order to eliminate any traces of contaminating genomic DNA.

For the reverse transcription and amplification of LjCAα – transcripts, Qiagen One Step RT-PCR system (Qiagen GmbH, Hilden, Germany) was used.

For the genes in study, 1μg of total RNA (100 ng) was reverse transcribed using a pair of gene-specific designed primers.

The primers used for the amplification of cDNA clones of the LjCAα1-type and LjCAα2-type respectively were:

LjCAα1 - F (5'- GAGAGCTGTTATTGGAATATGG -3')
LjCAα1 - R (5'- ACAAGTTCATCATCGTCCTAGG - 3')
LjCAα2 - F (5'- CTTGACCCCTGATAACAATGGC - 3')
LjCAα2 - R (5'- CCTATGGTTGTAATGGTCATAC -3')

Reactions were run on a Gene Amp PCR system 9600 (Perkin Elmer) for 35 cycles of 95°C (15 min), 54°C (1 min) and 72°C (1 min).

The RT-PCR products were analyzed by 1.5% agarose gel electrophoresis blotted on nylon membrane and hybridized to digoxigenin 11-rUTP-labelled inserts of LjCAα1- and LjCAα2-type cDNA clones.

Membrane hybridization (16h) and washing (twice for 15 min in 2XSSC, 0.1% SDS and then 15 min in 0.1 XSSC, 0.1% SDS) were performed at 62°C according to standard protocols (Southern, 1975; Sambrook et al., 1989).

## RESULTS AND DISCUSSIONS

To gain insight into the carbon metabolism in various tissues of Lotus *japonicus* L., the accumulation of two LjCAα-type transcripts in different *L japonicus* L. tissues (nodules, leaves, stems, roots, flowers, green seedpods, germinated hypocotyls, germinated cotyledons, and apical meristems) were examined (LjCAα1- and LjCAα2).

Plants used for the characterization of carbonic anhydrase expression were grown in Holland nutrient solution (Flemetakis et al., 2002).

For this purpose, total RNA was isolated from different *Lotus japonicus* tissues. A semi quantitative reverse-transcription (RT)-PCR approach employed.

1µg of total RNA (100 ng) treated with DNAse was used for each reaction, and the different RNA preparations were normalized by parallel amplification of the constitutively expressed gene LjUbiquitin using LjUBQ-F (5'–ATGCAGATCTTTTGTGAAGAC-3') and LjUBQ–R (5'-ACCACCACGGAAGACGGAG-3') primers.

Under these experimental conditions, an exponential increase of the amplification products was observed until after 35 amplification cycles (94°C/1 min, 54°C/1 min, 72°C/1 min). All the amplification reaction was performed under these conditions in order to obtain semi-quantitative results.

The accumulation of LjCAα1-type mRNA was observed in mature nodules and also in flowers and green seedpods. The highest level of mRNA was observed in sink tissues such as nodules. Relatively lower levels were found in green seedpods. For all others analyzed tissues no transcripts were detected.

The LjCAα2-type transcripts were detected in all analyzed tissues (nodules, leaves, stems, roots, flowers, green seedpods, germinated hypocotyls, germinated cotyledons and apical meristems). The results indicate that gene’s transcripts were at varying levels in different plant tissues, no matter if they were symbiotic or non-symbiotic organs. The highest level of mRNA was observed also in sink tissues such as nodules (Figure 1).

We can conclude that each of the analyzed CA genes may exhibits diversity of function, even they belong to the same α–class. These genes may be as well as examples of stringent preservation.

## CONCLUSIONS

The accumulation of LjCAα1 mRNA was observed in mature nodules and also in flowers and pods. The highest level of mRNA was observed in sink tissues such as nodules. Relatively lower levels were found in green seedpods. For all others analysed tissues no transcripts were detected.

The data showed that LjCAα2-type transcripts were detected in all analysed tissues (nodules, leaves, stems, roots, flowers, green seedpods, germinated hypocotyls, germinated cotyledons, and apical meristem). The results indicate that gene’s transcripts were at varying levels in different plant tissues, no matter if they were symbiotic or non-symbiotic organs. The highest level of mRNA was observed also in sink tissues such as nodules.

The levels of carbonic anhydrases expression vary with the type of the gene under study, even they belongs to the same genetic class.

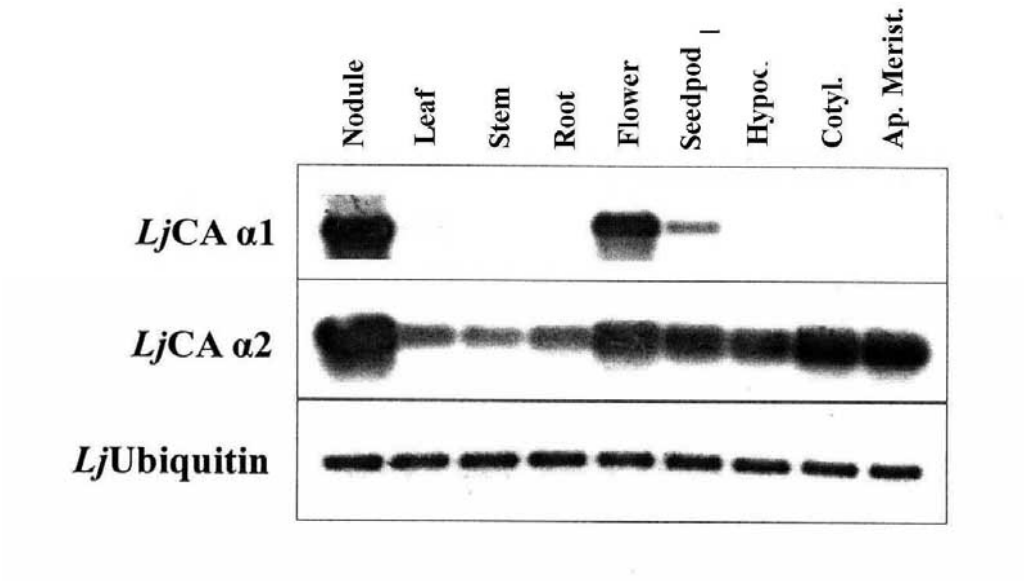


**Figure 1. Accumulation of LjCAα1 and LjCAα2 -type transcripts in symbiotic and non symbiotic tissues of *Lotus japonicus* L. (Gifu B-129). Total RNA was isolated from various tissues as indicated and subjected to semi-quantitative RT-PCR analysis using *L. japonicus* L. ubiquitin as an internal control.**

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