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

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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 anhydrases genes (LjCA α-type and LjCA β-type) was determined in different organs of *Lotus japonicus* L.

INTRODUCTION

Carbonic anhydrase (CA) is a primitive and ubiquitous enzyme found in virtually every tissue and cell type, in many subcellular organelles and in organisms ranging from unicellular cyanobacteria through mammals (Badger and Price, 1994; Dodgson et al., 1991; Maren, 1967). The enzyme catalyzes the reverse hydration of CO_2 to bicarbonate. The reaction underlying many diverse physiological processes in animals, archaebacteria and eubacteria.

All carbonic anhydrases are divided into three genetically distinct classes (α, β and γ) that have no sequence homology and evolved independently (Hewett-Emmet and Tashian, 1996). 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.

Carbonic anhydrates provide an excellent example of convergent evolution.

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).

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 INVESTIGATIONS

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

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 a 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 analysis

To analyze carbonic anhydrase genes expression, total RNA was isolated from different *Lotus japonicus* tissues (nodules, roots, leaves, stems, flowers, green seedpods, germinated cotyledons, germinated hypocotyls and apical meristem), according to 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 LjCA transcripts, Qiagen One Step RT-PCR system (Qiagen GmbH, Hilden, Germany) was used. For the genes in study, 1μg of total RNA (100ng) was reverse transcribed using designed primers. The primers used for the amplification of cDNA clones of the LjCA-α-type and respectively LjCA-β type were:

LjCAalpha-type–F (5'-GAGAGCTGTTATTGGAATATGG-3')

LjCA-alpha-type–R (5'- ACAAGTTCATCATCGTCCTAGG- 3')

LjCAbeta-type–F(5'-AGCTGAGGTACAGTGGAGTAGG-3')

LjCA-beta type–R (5'- TGAAGGTAGTGCCATGCTTCAG- 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 LjCA-α-type and LjCA-β-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 LjCA α-type and Lj CA β-type transcripts were examined.

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 (nodules, roots, leaves, stems, flowers, green seedpods, germinated cotyledons, germinated hypocotyls and apical meristem). A semiquantitative reverse-transcription (RT)-PCR approach employed.

1μg of total RNA (100ng) treated with DNAse was used for each reaction, and the different RNA preparations were normalized by parallel amplification of the constitutivelly expressed gene LjUbiquitin using LjUBQ-F(5'–ATGCAGATCTTTTGTGAAGAC-3') and Lj UBQ–R(5'-ACCACCACGGAAGACGGAG- 3') primers. Under our experimental conditions, an exponential increase of the amplification products was observed until after 35 amplification cycles. All the amplification reaction was performed under these conditions in order to obtain semi-quantitative results.

The accumulation of Lj CA-α mRNA was observed in mature noduls 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 Lj CA-β transcripts were detected only in nodules, where the abundance of transcript was considerably high. This result might suggest that LjCA-β is a true nodulin gene.

In all the other tested tissues, no hybridization signal was observed. (Figure 1).

We can conclude that each of the CA gene families exhibits diversity of function as well as examples of stringent conservation, yet the similarity of their catalytic mechanism is a remarkable example of convergence evolution.

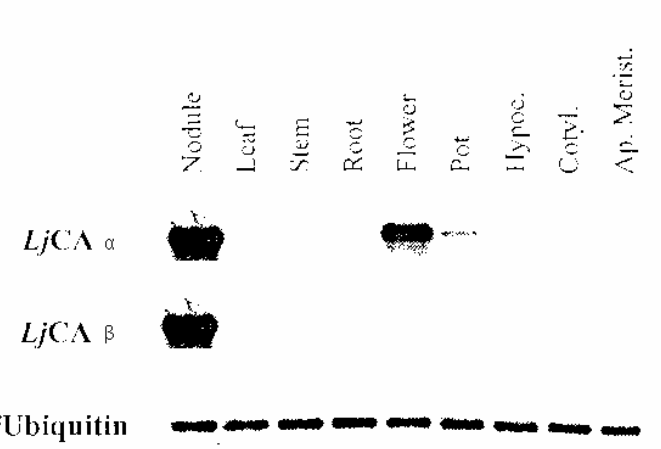

Figure 1. Accumulation of LjCA α-type and LjCA β-type mRNAs in *Lotus japonicus* L. non-symbiotic tissues. Total RNA was isolated from various tissues as indicated and subjected to semi-quantitative RT-PCR analysis using L. japonicus ubiquitin as an internal control.

CONCLUSIONS

The accumulation of Lj CA-α mRNA was observed in mature noduls 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 LjCA beta type transcripts appeared to accumulate at high levels only in nodules. In all the other tissues tested, no hybridization signal was observed.

The levels of carbonic anhydrases expression varies with the type of the gene under study.

BIBLIOGRAPHY

Badger, M.R., Price, G.D., 1994. The role of carbonic anhydrase in photosynthesis. Annu. Rev. Plant. Physiol. Plant Mol. Biol. 45:369-392

Brusslan, E and Tobin, C., 1992. Isolation of nucleic acids. PNAS 89:7791-7795

Chollet, R., Vidal, J., O'Leary, M.H., 1996. Phosphoenolpyruvate carboxylase: A ubiquitous highly regulated enzyme in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47:273-298

Dodgson, S.J., Tashian, R.E., Gros, G., Carter, Nd eds, 1991. The Carbonic Anhydrases. Cellular Physiology and Molecular Genetics. New York. Plenum. P. 379

Flemetakis E., A. Agalou, N. Kavroulakis, M. Dimou, A. Martisikovskaya, A. Slater, H.P. Spaink, A. Roussis and P. Katinakis, 2002. Lotus japonicus gene Ljsbp is highly conserved among plants and animals and encodes a homologue to the mammalian selenium – binding proteins. Molecular Plant – Microbe Interactions, Vol. 15, No.4, p. 390-403

Handberg K. and Stougaard, J., 1992. Lotus japonicus, an autogamous, diploid legume species for classical and molecular genetics. Plant Journal 2: 487-496

Henry, R.P., 1996. Multiple roles of the carbonic anhydrase in cellular transport and metabolism, Annu. Rev. Physiol. 58: 523-38

Hewett-Emmet and Tashian, 1996. Functional diversity, conservation and convergence in the evolution of the α-, β- and γ-Cas families. Mol. Phylogenet. Evol. 5:50-77

Kimber, M.S. and Pai, E.F., 2000. The active site arhitecture of Pisum sativum β-carbonic anhydrase is a mirror image of that of a α-carbonic anhydrases. EMBO J., 19: 1407-1418

Kisker et al., 1996. A left-hand β-helix revealed by the crystal structure of a carbonic anhydrase from the Methanosarcina thermophila. EMBO J. 15: 2323-2330.

Maren, T.H., 1967. Carbonic Anhydrases:chemistry, physiology and inhibition. Physiol. Rev.47: 595-781

Okabe, K., Lindlar, A., Tsuzuki, M.& Miyachi, S., 1980. Effects of carbonic anhydrase in ribulose-1,5-bisphosphate carboxylose and oxygenase – FEBS Lett. 114:142-144

Mitsuhashi et al., 2000. X-ray structure of β-carbonic anhydrase from red alga Porphyridium purpureum reveals a novel catalytic site for CO2 hydration. J. Biol. Chem. 275: 5521-5526

Rosendal, L., Vance, C.P., Pedersen, W. B., 1990. Products of dark CO2 fixation in pea root nodules support bacteroid metabolism. Plant. Physiol.93;12-19

Sambrook, J., Fritsch, F.F., Maniatis, T., 1989. *Molecular Cloning. A Laboratory Manual,* 2 nd ed. Cold Spring Harbor laboratory, Cold Spring Harbor, N.Y.

Southern, E., 1975. Detection of specific sequences among DNA frragments separated by gel electrophoresis. I. Mol. Biol. 98: 503-507

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