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Cloning and characterization of the genomic sequence and promoter region of pea chloroplastic fructose-1,6-bisphosphatase

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Introduction

Chloroplastic fructose-1,6-bisphosphatase (FBPase) is an essential enzyme for carbohydrate synthesis during photosynthesis. One of its most interesting feature is its activation during the illumination through a light modulated reduction of a -S-S- bridge located in the enzyme via the ferredoxin-thioredoxin system (Csèke et al. 1986). The electron transfer from ferredoxin to thioredoxin (Trx) is catalysed by ferredoxin-thioredoxin reductase. Chloroplastic FBPase is specifically activated by thioredoxin f, even though the pea enzyme can also be activated by Trx m (Lopez et al, 1997). The cytosolic version of plant FBPase is involved in the sucrose synthesis and is inhibited by AMP and fructose-2,6-bisphosphate. Despite the high conserved regions found between the amino acid sequences of both enzymes, the phylogenetic tree built up with FBPases from different species arranges plant cytosolic and chloroplastic FBPases in separate groups. The main difference among them is an extra region of the chloroplastic FBPase, which support two cisteines, involved in the redox regulation. As seems with other enzymes of the Calvin cycle, the synthesis of chloroplast FBPase is also activated by light showing its promoter region "light responsive elements" (LRE), involved in the light induction of the enzyme (Arguello et al. 1998). In order to understand some of the regulatory properties of plant FBPases, and to identify the LRE of the chloroplast enzyme, we have isolated and characterized the genomic sequence and the promoter region of pea chloroplastic FBPase.

Material and Methods

Phylogenetic tree was built up with the amino acid FBPase sequences from different sources available in GenBank and Protein Identification Resource (PIR), using the DARWIN computer program.

We have amplified a 1.6 kb genomic DNA sequence of the pea chloroplastic FBPase by.using the polymerase chain reaction (PCR), and N-terminal and C-terminal oligonucleotides pair, designed from the earlier known FBPase cDNA sequence (Carrasco et al. 1994), After agarose gel electrophoresis, the isolated band was cut from the gel, purified and subcloned into the pGEM-T vector (Promega). This pFgA clone was sequenced in both directions with an automatic sequencer using fluorescent dye terminator.

For the isolation of the chloroplastic FBPase promoter region we have used the PCR walking method (Devic et al. 1997), an improved PCR method for walking on uncloned genomic DNA. The CTAB method (Doyle et al. 1990) was used to obtain a small scale pea genomic DNA preparation. To this end we constructed 7 libraries by digestion with EcoRV,

ScaI, DraI, PvuII, SspI, HpaI, StuI, and ligation of adaptor oligonucleotides. We have designed primers for the N-terminus of the FBPase enzyme, FBP1 (24 mers) and FBP2 (25 mers) both in upstream direction, and nested primers AP1 (27 mers) and AP2 (18 mers) homologues to the adaptors. The PCR reactions were performed in 2 steps. The first PCR was carried out with the couple FBP1/AP1 and each library; after dilution of the first PCR products, FBP2/AP2 was used for a second PCR. The bands of interest were isolated from the agarose gel, eluted and ligated into pGEM-T (Promega). The plasmids were sequenced with an ABI automatic sequencer using fluorescent dye terminator.

Total RNA was prepared from leaves of *Pisum sativum,* 20 and 40 µg subjected to primer extension using 10 pmol of synthetic oligonucleotide, 5' end-labelled with γ- ^{32}P dATP, and polynucleotide kinase (Amersham-Pharmacia). The extension product was resolved by urea/polyacrylamide gel electrophoresis. Size determination of the fragments was performed by comparison with the dideoxy sequencing of FBPase promoter gene.

Results and Discussion

Using the DARWIN program, we constructed a phylogenetic tree by comparing the FBPase amino acid sequences (Fig.1) available from the GeneBank and Protein Identification Resource (PIR).

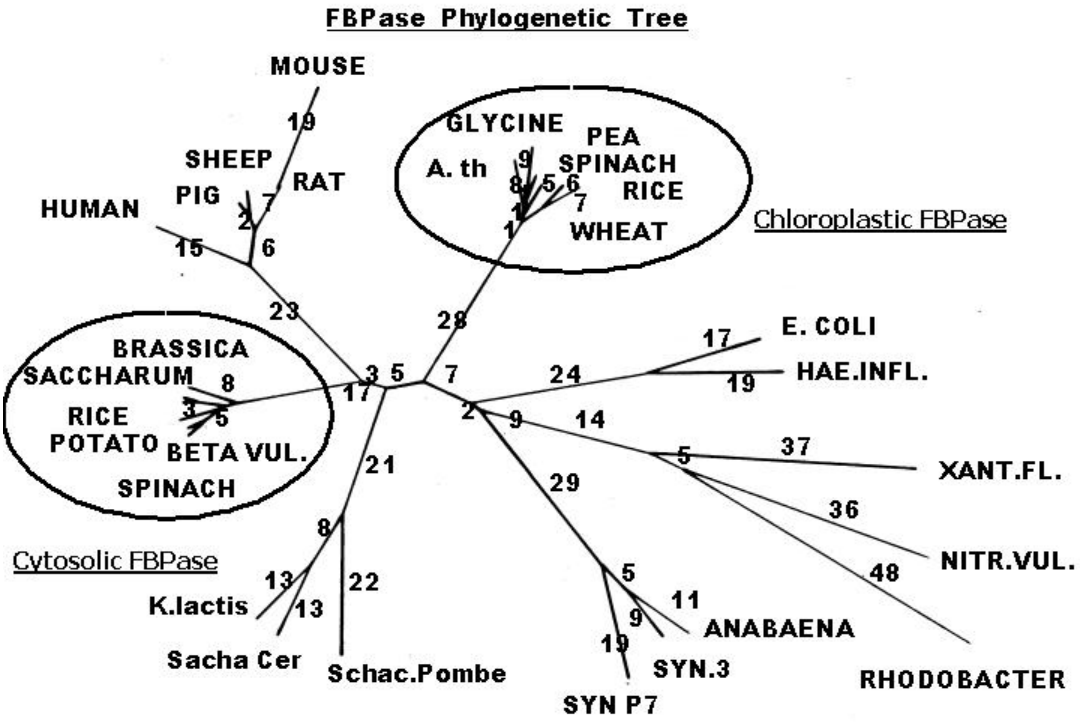

Fig.1. DARWIN tree of FBPase proteins from different sources

Plant fructose-1,6-bisphosphatases appears organized in two separate groups, chloroplastic and cytosolic. FBPases from vertebrates, yeast and prokaryotes are also classified in different groups. This indicates that FBPases, although probably comes from one common ancestor, have diverged within the different eukaryotes along the evolution. However, the alignment of the amino acid sequences of FBPase from plants (chloroplastic and cytosolic) and vertebrates (Fig 2), shows up to 75% highly conserved regions. A noteworthy exception is an extra region of the chloroplastic FBPase, which supports three cisteines (153,173 and 178 in the pea

enzyme), two of them (153 and 173) involved in the formation of the -S-S- bridge responsible of redox regulation of the chloroplast enzyme.

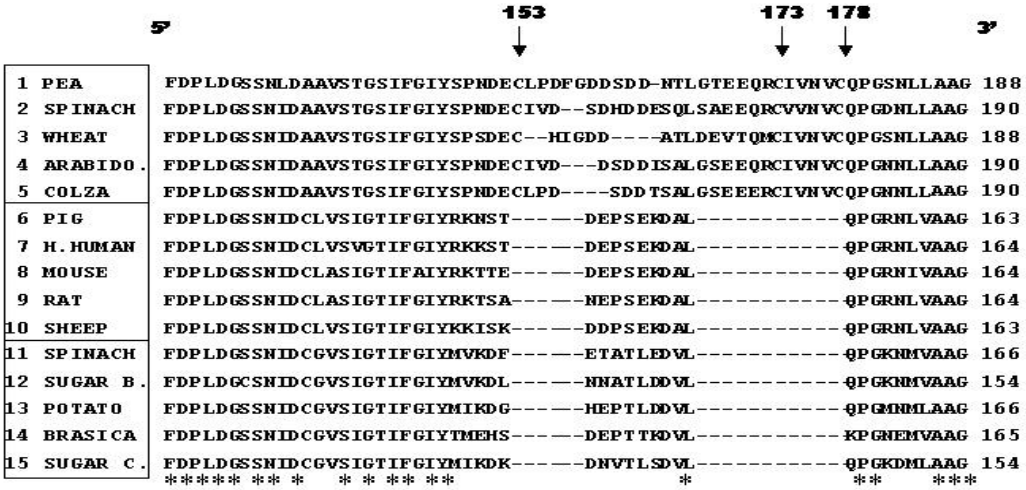

Fig.2. Alignment of the FBPase regulatory regions

The analysis of the pea chloroplastic FBPase genomic DNA sequence shows 4 open reading frames (ORFs), and 3 introns of 206 bp, 146 bp, 256 bp. Intron positions were determined by comparison with the cDNA sequence. The 5' splice junctions and the putative branch points of all three introns correspond to the consensus sequences (G/GT) of introns from nuclear genes in higher plants (Sinibaldi et al. 1992). The 3' splice junctions are not as well conserved. Interestingly, the 3 intron positions are conserved with respect to those of wheat (Lloyd et al. 1991) and *Arabidopsis thaliana* chloroplastic FBPase sequences (Fig.3). On the contrary, we could not find similarities in the size and sequence of the different introns.

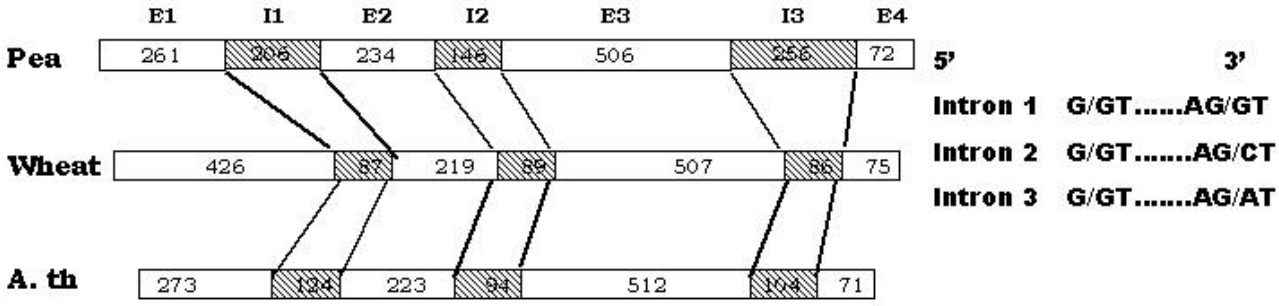

Fig.3. Intron positions of the pea, wheat and Arabidopsis genomic DNA of chloroplastic FBPases

This result also suggest a common ancestor for chloroplastic FBPase. When the analysis was extended to the genomic DNA of *A. thaliana* cytosolic FBPase (data base), the nucleotide sequence showed the presence of 10 introns, and no one of them coincide in position with those of the chloroplastic genomic DNA. This supports a clear situation of divergence along the evolution of plant FBPases.

As conclusion, these results seems to show that all the FBPases have the same ancestor in their origin. The divergence within the plant enzyme probably appeared with the introduction of the redox regulatory region in the chloroplastic enzyme. Despite the different theories about the role of intron in evolution, we suggest that introns were introduced after the divergence and division in the genes of chloroplastic and cytosolic FBPases.

By using the PCR walking method we have isolated several bands from the different pea genomic DNA libraries. We subcloned them in pGEM-T and sequenced the putative interesting fragments. To this end we choose pFBP17 and pFBP18, which have the region corresponding to the promoter region of the pea chloroplastic FBPase. The computer analysis of this sequences with the program PLACE showed several interesting regulatory elements: (Fig 4). GATA motif, CAAT box and I boxes (GATAA), which have been earlier described as "light responsive elements" (LRE) in different light-dependent genes.

Fig.4. Light responsive elements (in dark) in the pea chloroplastic FBPase promoter

This strengthens the previous assumption of light-regulated expression of pea chloroplastic FBPase. Our future aim is to localyze the cis-acting elements required for the light regulated expression of the enzyme. Actually we are preparing different constructions containing the whole pea promoter region and individual LRE fused to a reporter gene, in order to introduce them in *A. thaliana* plants.

The primer-extension method have determined one intense band. We concluded that the transcription of the pea chloroplastic FBPase gene might started at the position -24 bp upstream from the initiation codon. However some more experiments has to be made to confirm this data.

References

Cséke et al. (1986).*Biochem.Biophys.Acta* **853**: 43-63
López-Jaramillo et al. (1997). *Plant Physiol.* **114**: 1169-1175
Arguello et al. (1998). *Ann.Rev.Plant Physiol.Plant Mol.Biol.* **49**:525-555
Carrasco et al. (1994). *Planta* **193**: 494-501
Devic et al.(1997). *Plant Physiol. Biochem*. **35**: 331-339
Doyle and Doyle (1990). *Focus* **12**: 13-15
Sinibaldi et al (1992) *Prog Nucleic Res Mol Biol* **42**: 229-257.
Lloyd et al. (1991). *Mol Gen Genet* **225**: 209-216