***Cyanophora paradoxa*:**

Nucleotide Sequence and Phylogeny of the Nucleus Encoded Muroplast Fructose-1,6-bisphosphate Aldolase

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Immunoscreening of a *C. paradoxa* expression library against water soluble muroplast ("cyanelle") proteins resulted in isolation of a clone encoding the nucleus-encoded muroplast class-II fructose-1,6-bisphosphate aldolase (class-II FBA^s). Its nucleotide sequence was determined. The 1432 bp insert, derived from a single-copy gene transcript, bears a reading frame of 1206 bp in length, representing 402 amino acids with 346 amino acids of mature protein. The leading amino acids match structural features necessary for precursor import across chloroplast envelope membranes. In phylogenetic tree topology, the investigated mature FBA clusters within type B FBAs with *Synechocystis* sp. as nearest neighbor. This is the first report of a Type B class-II FBA sequence of plastids.

Introduction

Being part of fundamental pathways for conservation and utilization of energy and metabolites fructose-1,6-bisphosphate aldolase (FBA, EC 4.1.2.13) is present in most living organisms (Horecker *et al.*, 1972; Plaumann *et al.*, 1997). In glycolysis and gluconeogenesis it is the key tool at the hexose-triose-transition (Embden-Meyerhof pathway), where it is catalyzing the cleavage of fructose-1,6-bisphosphate (FBP) into triose-phosphates or vice versa, depending on metabolic situations. Within the Calvin-Benson cycle, aldolase plays a similar role preferentially leading the trioses towards hexose sink and CO₂-acceptor regeneration.

Two distinct species of FBAs, designated class-I and class-II, have been characterized (Jacobsha-

gen and Schnarrenberger 1988; Marsh and Lebherz 1992). All known FBAs share a homomer complex structure: Class-I aldolases are tetramers with a total molecular weight of about 160 kDa, whereas the native form of class-II aldolases is dimeric with a molecular weight of about 78 kDa. Although they are catalyzing the same reaction, class-I and class-II aldolases differ in their complex phylogenetic distribution (Schnarrenberger, 1985) across taxonomic groups: their amino acid sequences, the structure of their active sites (Marsh and Lebherz, 1992) and the reaction mechanisms (Rutter, 1964; Morse and Horecker, 1968; Mildvan *et al.*, 1971). In some species even both types are present (Marsh and Lebherz, 1992).

Class-I aldolases are found in green algae, higher plants (Schnarrenberger *et al.*, 1990) as well as in animals (Sygusch *et al.*, 1987) and less frequently in bacteria (Baldwin and Perham, 1978). The class-II aldolases are coded for in bacteria (Alefounder *et al.*, 1989), fungi (Schwelberger *et al.*, 1989), *C. paradoxa* (Gross *et al.*, 1994), and *Giardia intestinalis* (Henze *et al.*, 1998). In *C. paradoxa* the muroplast class II enzyme additionally

^s Abbreviations: class-II FBA, class-II fructose-1,6-bisphosphate aldolase; cTPs, chloroplast transit peptides; FBP, fructose-1,6-bisphosphate; *groEL*, heat shock protein; IPTG, Isopropylthiogalactosid; *petF*, ferredoxin-NADP⁺-oxidoreductase; *psaE*, subunit of PS-I complex; *psaF*, subunit of PS-I complex.

shows, as other plastidic multifunctional class I aldolases, a comparable sedoheptulose activity (Flechner *et al.*, 1999).

Phylogenetic analysis of class-II FBAs led to separation of two types, designated type A and type B class-II FBA originating from gene duplication (Plaumann *et al.*, 1997).

Discussing endosymbiosis theory in view of chloroplast evolution, it remains as yet an unclear and intriguing question whether *C. paradoxa* is an anagenetic model organism indicating the idea of a polyphyletic origin of primary plastids. Or a descendant of the missing link in the evolutionary chain to the protoplastid originating from the only endocytobiotic cyanobacterium having been able to undergo intertaxonic combination with its unique eukaryotic host.

The flagellate *C. paradoxa* normally bears two to four chromatophoric bodies. From the structural point of view, including the surrounding murein sacculus (Schenk, 1970), these bodies seem to be still intracellular cyanobacteria (Pascher's cyanelles, 1929). Considering the genomic level reveals a genome that is reduced (Herdman and Stanier, 1977) and structured like higher plant plastomes (Löffelhardt *et al.*, 1980). Furthermore there are reports of protein import into this cell compartment (Bayer and Schenk, 1986; Bayer *et al.*, 1990; Jakowitsch *et al.*, 1996) together with the first direct proof of gene transfer from the originally endocytobiotic cyanobacteria's genome into the nucleus of its host (Schenk *et al.*, 1992).

That's why we assigned to the glaucocystophytan "cyanelles" the status of a real cell organelle and altered their term into cyanoplasts (Schenk, 1977; 1990), also known as muroplasts (Schenk, 1994; Löffelhardt *et al.*, 1999). Further analysis of single gene content of the *C. paradoxa* plastome (Löffelhardt *et al.*, 1997a) revealed a set of genes which are no longer plastome-based in higher plants (e.g. *psaE*, *psaF*, *petF*, *groEL* and several ribosomal proteins). In view of a monophyletic evolution of plastids, *C. paradoxa* muroplasts may therefore be considered as a late stage of the postulated protoplastid (Löffelhardt *et al.*, 1997b).

For *C. paradoxa* two isoforms of the nuclear encoded class II FBA, a cytosolic and a muroplastidic protein, have been identified by their different biochemical and biophysical properties (Gross *et al.*, 1994; Flechner *et al.*, 1999). In this

paper we present the sequence for a nuclear coded type B class-II fructose-1,6-bisphosphate aldolase from *C. paradoxa* and provide indications that this protein is the muroplast's enzyme. The phylogenetic implications based upon fba compartmentation and fba type are discussed.

Materials and Methods

Strain and culture conditions and preparation of antibodies

C. paradoxa (LB 555 UTEX, Pringsheim strain B 29.80 from SAG, Culture Collection of Algae, Göttingen, Germany) was grown and harvested according to Zook and Schenk (1986). To meet the specific needs of detecting unprocessed and mature proteins in various gel systems, polyspecific polyclonal antibodies were prepared in rabbits and purified over Protein A-Sepharose as described by Bayer (1991). Water soluble muroplast and membrane hosted proteins for immunizing were obtained according to Zook and Schenk (1986) and Bayer and Schenk (1989).

Construction of cDNA library and screening of cDNA library

Total RNA was isolated from a *C. paradoxa* culture 24 hours after dilution by the guanidinium thiocyanate method described in the "The Qiagenologist" application protocols (3rd. Ed., Qiagen, Hilden, Germany, 1990). The poly(A)⁺-RNA fraction was separated from total RNA by purification over OligotexTM-dT30 (Qiagen) and the cDNA library was constructed using the Uni-ZAP[®] synthesis kit (Stratagene, La Jolla, Ca., USA) following the suppliers instructions and amplified prior to screening.

Phages (10⁵ pfu) were incubated at 37 °C for 15 min in 100 µl *E. coli* XL1-Blue (OD₆₀₀=2.0) and cultured by the top-agar technique under 100 µg ml⁻¹ ampicillin selection and induction by 10 mM IPTG. When the plaques reached about 0.5 mm in diameter after for 3 to 4 h, expressed proteins from lysed cells were transferred to nitrocellulose membranes. Immunodetection was performed according to the instruction manual of the ECL-western blotting detection system (Amersham Pharmacia, Freiburg, Deutschland) and posi-

tive plaques were rescreened until all plaques per plate were positive.

Recombinant phages were inserted into Bluescript SK(minus) plasmids by the “ λ -ZAP-Automated Excision Process” using the F1-ExAssistTM helper phage (Short *et al.*, 1988) and subclones were generated according to Henikoff (1984) using the Nested Deletion Kit from (Amersham Pharmacia, Freiburg, Deutschland).

Polymerase chain reaction (PCR)

The ABI Prism Dye Terminator Cycle Sequencing Kit with AmpliTag[®] Polymerase (Perkin Elmer, Foster City, CA., USA) was used to perform PCR using a Biozym Minicycler[®].

DNA sequencing and sequence analysis

Clones were sequenced by the dideoxynucleotide chain-termination method of Sanger *et al.*, (1977) using an automated DNA-analyzer (ABI Model 320).

The following oligonucleotides were used as primers:

- (a) 5'-ATCAAGTCGATCATGGAGGC-3'
and
(b) 5'-CTTCTGCATGTACTTCATCG-3'.

Using the HUSAR/GCG package (maintained by the Biocomputing Unit at DKFZ Heidelberg, Germany) and the BLAST Server (NCBI, Bethesda, MA., USA) the sequence was subject to further analysis. Alignment of amino acid sequences were based upon algorithms from the ClustalX 1.64 /ClustalW 1.7.4 package (Thompson *et al.*, 1994; Thompson *et al.*, 1997), hydropathy plots of transit peptides – using the Kyte-Doolittle scale (1982) – and amino acid frequency calculations were performed with the DNASTar-package (Lasergene Inc., Madison, WI., USA). Phylogenetic trees were constructed with the ClustalW 1.7.4 program using Dayhoff matrices for calculating NJ-distances. Bootstrap analysis was carried out with 1000 replicates.

Estimation of gene copy number

Gene copy numbers were estimated by Southern hybridization of noncutler-treated genomic DNA with an internal 808-bp-fragment (Fig. 3), la-

Fig. 1. Northern blot analysis. 2 μ g *C. paradoxa* mRNA were fractionated by electrophoresis under denaturing conditions with reference to a 0.24–9.5 kb RNA ladder (GibcoBRL, LifeTechnologies, Germany). The mRNA was blotted onto Qiabrine Nylon Plus membranes and hybridized with an α -[³²P]-labeled 808 bp internal *Xho* I fragment of class-II FBA cDNA.

Fig. 2. Southern blot analysis. *C. paradoxa* genomic DNA (10 μ g per lane) was digested with *Xho* I (lane 1), *Sal* I (lane 2), and *Sac* I (lane 3) and separated on a 0.7% agarose gel. The DNA was transferred to a Qiabrine Nylon Plus membrane and probed with an α -[³²P]-labeled 808 bp internal *Xho* I fragment of class-II FBA cDNA. Lambda *Eco* 47III DNA fragments were used as molecular size markers.

beled with α -[³²P]-dCTP (ICN, Costa Mesa, Ca., USA) by the random prime labeling method (Feinberg and Vogelstein, 1983) using a kit from MBI Fermentas (Vilnius, Lithuania).

Results

Identification of transcripts in vivo and final cellular location of the mature FBA

Screening of the *C. paradoxa* cDNA library yielded a set of clones positive to a polyspecific antiserum against water soluble muroplast proteins. Subject to further investigation was a clone carrying a 1432 bp insert (see below), which was probed against *C. paradoxa* mRNA in Northern analysis (Fig. 1). This revealed a single band of 1520 bp, therefore the fragment captured seems to be about full length.

Southern blot analysis was performed to determine the number of FBA genes in the *C. paradoxa* genome. The blots were probed with the *Xho* I-fragment under high-stringency conditions, and yielded for *Xho* I and *Sac* I digestions one signal each, accounting for one single gene locus (Fig. 2). The double band observed on the *Sal* I lane originates from the *Sal* I site at position 1174 of the FBA gene (Fig. 3).

Sequence analysis

Sequence identification. Sequencing found the insert to be 1432 bp long (Fig. 3) with a reading frame for a polypeptide of 402 amino acids. Its deduced amino acid sequence in comparison to known FBA sequences led to the following findings:

The sequence positions 56 to 402 contain the complete sequence of a mature class-II FBA with amino acid sequence homology to the known class-II fructose-1,6-bisphosphate aldolases of up to 71% similarities with the *Synechococcus* PCC6308 protein (Table I).

A high degree of conservation (Fig. 4), particularly in domains crucial for enzyme activity (Fig. 3: metal binding sites His¹³⁶, His¹³⁹) as known from three-dimensional structure analysis of the *E. coli* enzyme (Blom *et al.*, 1996, Cooper *et al.*, 1996).

The same inserts that match full helical turns as the other FBAs residing in the same subclade (Fig. 4, region A to C) and other sequence features accounting for surface property patterns like hydrophobic or strongly charged positions in phase with helical turns (Fig. 4, region D and E: bold letters).

Although being incomplete, the leading stretch of amino acid pos. 1–55 can be assigned the function of a transit peptide. It shares important features (von Heijne *et al.*, 1989) with other chloroplast transit peptides (cTPs) crossing the two envelope membranes, e.g. amino acid composition, extended β -strand structures (not shown) and the hydrophathy profile (Fig. 5).

Processing-site identification and mole mass. Comparison of the deduced protein with chloroplast aldolases from different species suggests an N-terminus for the mature monomer at Ala¹ (Fig. 3). Processing would yield a mature protein with a predicted molecular mass (M_r) of 37.23 kDa. This is in good agreement with the molecular mass of 38 kDa previously determined for the cytosolic class-II FBA monomer of *Euglena gracilis* (Pelzer-Reith *et al.*, 1994) and 37 kDa for the *Ralstonia eutropha* enzyme (formerly *Alcaligenes eutrophus*: Schäferjohann *et al.*, 1995). Further support is given by matching the (-3,-1) rule (von Heijne, 1983, 1985). In contrast to the prokaryotes where in most cases Ala is observed in these two positions, no strict preference for Ala is found in the eukaryotes (Nielsen *et al.*, 1997). Therefore we suggest the cleavage site for FBA-cTP in *C. paradoxa* to be

... Glu-Val-Thr-Arg ↓ Met-Ala-Leu ...

with the N-terminal methionine probably to be removed by a stromal methionyl-aminopeptidase after initial cleavage of the cTP, as with other plastidic nucleus-encoded proteins (Ikeuchi *et al.*, 1989, Gavel and von Heijne, 1990).

Discussion

Phylogenetic considerations

Class-II FBA sequences have been reported from a variety of prokaryotic (e.g. Trach *et al.*, 1988; Alefounder and Perham, 1989; Schäferjohann *et al.*, 1995; Kaneko *et al.*, 1995) and eukaryotic (e.g. Schwelberger *et al.*, 1989; Mutoh and Hayashi, 1994; Plaumann *et al.*, 1997) species. Amino acid sequence alignments (Fig. 3, Table I) of *C. paradoxa* FBA with other class-II aldolases show a clustering with the cyanobacterial group and in toto moderate similarities (59–71%) to type-B of cyanobacteria and α - and β -proteobacteria (exception: *P. stutzeri*, a γ -proteobacterium) and then to

ggcacgag	CACCACGCCCCGGTCGTTATCTCCCGCACCGCCCCGTCGGTTGCGTCGAAGAAGTCGGAG	69
	T T A P V V I S R T A P S V A S K K S E	20
GTGTGCCAGGCCAAGGCCGGCTTCTCTGAAGGGCGGCCGCGCTCGGGTTCTTCTCTGAACAACGTTTCAG		138
V C Q A K A G F L K G G R R L G F F L N N V Q		43
GCCCCAAGAACAATGAGGGCGCCGAGGTCACCCGCATGGCGCTCGTTCCTCATGCGCATCTCTCTCGAC		207
A P K N N E G A E V T R M A L V P M R I L L D		66
CACGCCCGCGAGCACGGTACGGTCTCCCCGCGTTCAACGTGAACAACATGGAGCAGATCAAGTCGATC		276
H A A E H G Y G L P A F N V N N M E Q I K S I		89
ATGGAGGCCGCTAAGGCGACCAACTCGCCCGTCATCTCCAGGCGTCGCGCGGTGCCCGCTCGTACGCC		345
M E A A K A T N S P V I L Q A S R G A R S Y A		112
GGCGAGAACTTCTCTCCGCCACCTCATCTCGCCGCGCGGAGACCTACCCCGAGATCCCGATCGCCATG		414
G E N F L R H L I L A A A E T Y P E I P I A M		135
CACCAGGACCACGGCAACGGCCCGGCCACCTGCCTGTGCGCCATCCGCAACGGCTTCACCTCCGTCATG		438
H Q D H G N G P A T C L S A I R N G F T S V M		158
ATGGACGGCTCTCTCGAGGAGGACTCGAAGACCCCGCCAGCTACGAGTACAACGTTAACGTGACCAAG		552
M D G S L E E D S K T P A S Y E Y N V N V T K		181
ACCGTCTGCGACCTCGCGCACGCCGTCGGTGTGTCCGTGAGGGCGAGCTCGGCTGCCTCGGCAACCTC		621
T V C D L A H A V G V S V E G E L G C L G N L		204
GAGACCGGCCAGGGCGACAAGGAGGACGGCGTCGGCGCTGAGGGCGTCTCACCATCGAGCAGATGCTC		690
E T G Q G D K E D G V G A E G V L T I E Q M L		227
ACCGACCCGGCCAGGCCAAGGACTTCGTCGAGCGCACCAAGGTCGATGCCCTCGCCATCGCCATCGGC		759
T D P A Q A K D F V E R T K V D A L A I A I G		250
ACCTCCACGGCGCCTACAAGTCTCCCGCAAGCGGACGGCGCTATCTCCGCATGACCGCATCCGT		828
T S H G A Y K F S R K P D G A I L R I D R I R		273
GAGATCAACCAGGCGCTCCCGAACACCCACCTCGTCATGCACGGCTCGTCGTCCTCCGTCGGGAGGACCTG		897
E I N Q A V P N T H L V M H G S S S V P E D L		296
CTCGCCCTGATCAACGCCAACGGCGGCAACATCCCCGAGACTTACGGTGTGCCCGTTGAGGAGATCCAG		966
L A L I N A N G G N I P E T Y G V P V E E I Q		319
GCTGGCATCAAGGTGGTGTCCGCAAGGTCAACATCGACACTGACTGCCGCTTGCCATCTCCGCGGCC		1035
A G I K A G V R K V N I D T D C R L A I S A A		342
GTCCGTGAGGCGCTCGTCAAGGACACCAAGGAGTTTCGACCCCGCCATTCTCAAGCCTCGATGAAGTAC		1104
V R E A L V K D T K E F D P R H S Q A S M K Y		365
ATGCAGAAGGTCTGCGAGGACCGCTACGTGCAGTTCAACGCTGCCGCAACGCCGACAAGATCAAGGCC		1173
M Q K V C E D R Y V Q F N A A G N A D K I K A		388
GTCGACCTCGACCATGGCCAAGAAGTTCTACGCCACCGCGTAAATCTTCTCTCTGGCACCAGCGGGA		1242
V D L D T M A K K F Y A T A *		402
GGGCGCACGCCGCGGTCTCATTAGGGAGGCTCTAGCTTCCACCTCGGCTCAGGCGCTCTCCGTTCCCT		1311
TCTTGACAGTCTTAGCAGCAGTACGCGCCCGCTACGTGTACCATAGGTGGCCTCAACACTGGGGTTCGA		1380
AACTGTTAATGAAAAAACGACATACTGAAAAAATAAAAAAAActcgag		1432

Fig. 3. DNA- and amino acid sequence of *C. paradoxa* Type B class-II FBA. The arrow indicates the proposed site of precursor processing. The assumed first few residues of the mature protein are in bold face. The first eight nucleotides of the 5' end are the polylinker and the last six nucleotides of the 3' end are the *Xho*I cleavage site of pBluescript.

Table I. Relative amino acid similarities (%) of mature FBAs. Calculated with indels. Lower triangle shows the absolute number of identities by pairwise comparison. The sequence length is shown on the diagonal (See alignment Fig. 4).

	HAEIN	ECOLI	CAMJE	SCHPO	YEAST	EUGGR	CORGL	EDWIC	STRCO	NEUCR	BORBU	CYAPA	SYNP6	XANFL	RHOCA	RALEU	NOSCO	PSEST	NEIME	RHIME	GIAIN	THEAQ	DEIRA	HELPI	THEMA	TREPA	BACST	BACSU	MYCGE	UREUR	ARATH
HAEIN	359	71	62	51	49	49	40	69	37	49	53	16	15	16	15	16	16	17	17	15	15	18	18	15	15	17	17	17	18	18	15
ECOLI	256	359	63	48	46	48	36	86	37	47	59	17	17	16	15	17	17	17	17	16	14	17	17	15	15	15	16	17	18	17	17
CAMJE	226	227	354	51	48	47	38	62	36	45	53	20	19	19	17	19	20	20	18	17	22	19	18	17	18	20	19	20	20	17	
SCHPO	184	176	184	358	66	50	34	49	35	59	47	18	17	17	16	18	17	18	18	14	14	17	15	16	15	18	16	15	19	18	17
YEAST	179	170	173	240	359	53	33	46	34	59	44	18	16	18	16	17	17	17	16	15	15	17	16	16	17	17	16	16	20	19	17
EUGGR	184	178	176	186	197	363	35	50	35	48	45	17	16	18	16	18	17	18	17	14	14	17	16	16	15	18	17	17	17	17	17
CORGL	148	133	139	125	123	130	344	37	63	34	37	15	15	17	16	17	16	17	17	14	14	17	20	18	16	15	17	19	17	19	17
EDWIC	250	311	225	180	171	188	136	358	39	47	58	17	17	17	16	17	17	18	17	16	15	19	18	16	16	16	16	16	17	19	17
STRCO	136	137	132	129	128	131	222	143	343	35	35	16	16	16	15	17	17	17	17	15	15	18	18	15	16	17	20	17	20	20	17
NEUCR	183	176	168	219	220	181	130	176	132	366	43	15	15	16	14	15	15	16	17	15	15	18	17	16	16	17	18	17	18	18	18
BORBU	193	217	196	172	161	169	136	214	131	161	359	15	15	16	14	15	16	16	15	13	13	16	15	17	16	15	15	15	19	17	15
CYAPA	68	73	84	78	77	72	64	73	67	67	66	347	71	65	60	65	70	62	62	52	42	38	36	34	31	33	30	29	24	26	28
SYNP6	68	74	81	76	72	69	65	74	67	66	65	253	354	66	65	62	83	65	64	59	42	39	35	36	32	34	31	31	25	24	26
XANFL	70	71	82	75	78	79	70	74	68	73	68	231	235	354	65	67	67	71	66	60	42	39	38	36	33	38	31	30	26	25	27
RHOCA	68	68	73	68	71	68	67	68	64	62	60	213	233	231	354	62	63	62	58	69	42	35	35	34	30	33	28	28	24	23	23
RALEU	68	71	81	75	74	75	68	74	71	67	66	229	221	240	221	345	62	70	65	55	44	42	37	37	34	38	31	31	24	25	28
NOSCO	72	74	88	77	76	73	68	74	71	68	69	254	301	243	227	226	359	65	64	58	44	39	35	35	31	35	31	31	25	24	26
PSEST	74	74	85	78	75	77	73	77	70	72	70	220	233	252	223	248	236	354	77	57	44	39	38	34	34	36	31	30	25	25	27
NEIME	73	74	78	78	72	74	72	75	73	74	67	220	229	237	207	233	231	274	354	53	42	37	36	33	32	33	29	29	25	24	25
RHIME	66	73	73	64	66	64	60	69	64	68	59	190	215	216	251	199	211	208	194	359	41	35	33	31	29	31	28	26	23	23	23
GIAIN	62	57	71	58	64	60	65	62	61	64	54	147	149	149	151	152	158	157	150	148	323	43	44	39	40	40	42	38	28	27	30
THEAQ	72	68	85	70	68	69	77	76	70	75	65	134	141	140	126	145	141	140	131	127	143	305	64	51	51	40	45	47	33	31	35
DEIRA	72	70	75	62	65	67	71	73	70	69	60	126	126	136	124	130	128	138	129	121	146	197	305	48	49	39	45	43	32	28	32
HELPI	62	59	72	63	66	65	63	64	59	65	68	120	128	128	121	129	129	123	119	115	129	158	150	307	56	37	39	39	30	29	31
THEMA	61	61	68	60	70	60	60	64	61	67	64	114	117	123	111	122	117	124	119	109	134	162	157	179	315	37	42	40	34	31	31
TREPA	69	62	71	71	70	74	68	66	67	69	61	124	129	144	126	142	137	132	127	121	142	134	133	124	126	332	35	35	30	28	26
BACST	64	61	74	62	63	65	69	61	71	69	59	105	113	112	103	111	115	111	104	102	137	141	143	124	136	118	287	78	43	43	34
BACSU	65	64	70	57	61	64	64	65	63	65	59	104	112	110	101	111	115	110	105	97	126	146	135	122	128	117	224	285	44	41	34
MYCGE	67	68	75	73	75	66	70	73	71	70	73	88	91	96	87	86	93	94	92	85	96	107	104	97	110	102	128	130	288	51	33
UREUR	67	66	75	70	72	65	69	66	73	70	66	95	90	92	84	90	91	91	89	85	93	101	92	93	103	96	128	123	150	289	34
ARATH	59	65	64	65	64	65	61	65	61	69	59	99	95	100	84	99	97	100	93	87	101	111	101	99	103	88	101	101	99	102	278

Fig. 4. Alignment of class-II-FBA proteins. Gaps are indicated by dashes, shading at positions with more than 65% similarities (*) and identities (*). Sequences were retrieved from GenBank, Swissprot and PIR databases. Accession to sequences through index numbers is for *Arabidopsis thaliana* (ARATH), AAF25990.1, *Bacillus stearophilus* (BACST), P94453, *Bacillus subtilis* (BACSU), P42420, *Borrelia burgdorferi* (BORBU), O51401, *Campylobacter jejuni* (CAMJE), P53818, *Corynebacterium glutamicum* (CORGL), P19537, *Cyanophora paradoxa* (CYAPA), AJ227753, *Deinococcus radiodurans* (DEIRA), F75378, *Edwardsiella ictaluri* (EDWIC), O52402, *Escherichia coli* (ECOLI), P11604, *Euglena gracilis* (EUGGR), CAA61912, *Giardia intestinalis* (GIAIN), AF079109.1, *Haemophilus influenzae* (HAEIN), P44429, *Helicobacter pylori* (HELPI), C71967, *Hydrogenophilus thermoluteolus*, BAA75221.1, *Mycoplasma genitalium* (MYCGE), P47269, *Neisseria meningitidis* (NEIME), AAF42203.1, *Neurospora crassa* (NEUCR), P53444, *Nostoc commune* (NOSCO), Q9XDP3, *Pseudomonas stutzeri* (PSEST), O87796, *Ralstonia eutropha* (RALEU), Q59101, *Rhodobacter sphaeroides* (RHOCA), P29271, *Saccharomyces cerevisiae* (YEAST), P14540, *Schizosaccharomyces pombe* (SCHPO), P36580, *Sinorhizobium meliloti* (RHIME), P56888, *Streptomyces coelicolor* (STRCO), Q9X8R6, *Synechococcus PCC6803* (SYNP6), Q55664, *Thermotoga maritima* (THEMA), G72397, *Thermus aquaticus* (THEAQ), AAF22441.1, *Treponema pallidum* (TREPA), O83668, *Ureaplasma urealyticum* (UREUR), AAF31010.1, *Xanthobacter flavus* (XANMA), Q56815.

Fig. 4. Legend see on page 996.

Fig. 5. Hydropathy profiles for transit sequences (Kyte-Doolittle). Window size is 9 residues, positive (negative) peaks represent hydrophilic (hydrophobic) residues, arrows denote the putative cleaving site. Sequences were retrieved from GenBank, Swissprot and PIR databases. Accession to sequences through index numbers is for *C. paradoxa* FBA: AJ227753, *C. paradoxa* FNR: G1071829, *Triticum aestivum* GBSS: P27736, *Brassica napus* FBPase: Q07204, *Mesembryanthemum crystallinum* RbcS: Q08186, *Arabidopsis thaliana* rpl 12-C: P36212, *Spinacea oleracea* CP 12: Z72487, ditto Cyt b6f: S00454.

Fig. 6. Topology of gene tree for mature FBAs. Phylogenetic relations within the tested sequences (for data base accessions see legend to Fig. 4) by the neighbor joining method (Saitou and Nei, 1987), displayed with reference to substitution frequency per site (see scalebar). Probabilities for branching, as shown at the appropriate nodes were obtained through bootstrap analysis (Felsenstein, 1985) with 1000 replicates through the same distance estimation method. Eukaryotic species are marked by bold capital letters. The upper main branch includes the type B class-II FBA sequences, the lower those of the type A.

the other subgroups of the type-B sequences with similarity values of 24–38%. The next neighbor eukaryotes yet known are *G. intestinales* (formerly known as *G. lamblia*) with 42% and *A. thaliana* with 28% similarity. The cellular compartmentation and expression status of the *A. thaliana* polypeptide is not known. The FBA isozyme of *C. paradoxa* is more related to that of *Synechocystis* than to the FBA of any other known bacterial organism or eukaryote. There was low similarity (15–20%, Table I) to the class-II type A aldolases from γ -proteo- and other bacteria, fungi and *E. gracilis*.

Similarity-Distance-Analysis of the available FBA class II sequence data suggests gene duplication and subsequent metabolic specification of the related products designated type A and type B aldolases (Gross *et al.*, 1994; Plaumann *et al.*, 1997). Type A FBAs are functional in glycolysis and gluconeogenesis pathways, whereas normally type B FBAs belong to the Calvin-Benson-Cycle, clearly indicated by their gene loci clustering with (Alefouder and Perham, 1989; Schwelberger *et al.*, 1989; von der Osten *et al.*, 1989; Mutoh and Hayashi, 1994; Fleischmann *et al.*, 1995; Burucoa *et al.*, 1995; Plaumann *et al.*, 1997). This includes all investigated class-II FBA-genes found in the Calvin-Benson-Cycle operons of photoautotrophic proteobacteria and cyanobacteria (Meijer *et al.*, 1990; Chen *et al.*, 1991; Schäferjohann *et al.*, 1995; Kaneko *et al.*, 1995).

Sequence analysis and the high bootstrap support for the topology of the phylogenetic tree with the position of the *C. paradoxa* FBA in the type B subclade (Fig. 6) supports the view that the presented sequence is coding for the muroplast enzyme, which originated from transfer out of the endocytobiotic cyanobacterium while turning into the real cell compartment stage. This is a further direct proof for gene transfer (Schenk *et al.*, 1992).

We therefore assist the idea of Plaumann *et al.*, (1997) that a third independent origin of plastid FBA must exist. Considering phylogeny we should bear in mind that class I and class II FBA isozymes must have originated differently according to reaction mechanism and sequence diversity (Plaumann *et al.*, 1997).

Chlorophyta and embryophyta are found to have different class I FBA isozymes within the cytosol and their plastids with the original plastidic class II FBA gene thought to have been replaced

by his host's gene. A similar situation was found recently for the rhodophyte *Galdieria sulfuraria* (Gross *et al.*, 1999) which doesn't fit the observation of Ikawa *et al.*, (1972) that other investigated red algae contain class I as well as class II aldolase activity.

E. gracilis contains a plastidic class I FBA and a cytosolic type A class II isozyme, which is explained by secondary symbiosis of a plastidic class-I-bearing chlorophyte (Plaumann *et al.*, 1997). Class II FBA has been shown to exist in fungi (type A), including yeasts, and in few other unicellular eukaryotes like *E. gracilis* (type A), *G. intestinalis*, (type A), *T. vaginalis*, several marine planktonic algae and heterotrophic prokaryotes (type A or B). The *C. paradoxa* type B sequence is the first plastidic class II FBA known to date. On first sight this finding complies with the hypothesis of Gross *et al.*, (1999) on chloroplast evolution with transfer of cyanobacterial class II FBA to the host nucleus as first endocytobiotic step. In step 2 in green algae and higher plants (in *G. sulfuraria* and perhaps in the other red algae) this plastidic enzyme was replaced by a cytosolic class I FBA isozyme developing from the original cytosolic 'host' FBA by gene duplication. Would that be correct, then the cytosolic class II FBA in *C. paradoxa* might have been developed in a similar manner, e.g. following the described class II FBA gene transfer event (from cyanobacterial endocytobiont to eukaryotic host). If, in contrast, the cytosolic *C. paradoxa* class II FBA is original within this species, the monophyletic hypothesis for chloroplast evolution would be difficult to understand.

Referring to the *fba* gene as a tool to precise the position of *C. paradoxa* and the muroplast in the topology of the evolution and to back the challenge of the recent interpretation of the plastid evolution (Martin *et al.*, 1998) to be monophyletic the cytoplasmic *fba* isoenzyme needs to be typed on the genetic level.

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