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Cloning of the amphibolic Calvin cycle/OPPP enzyme D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) from spinach chloroplasts: functional and evolutionary aspects

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Abstract

Exploiting the differential expression of genes for Calvin cycle enzymes in bundle-sheath and mesophyll cells of the C4 plant *Sorghum bicolor* L., we isolated via subtractive hybridization a molecular probe for the Calvin cycle enzyme D-ribulose-5-phosphate 3-epimerase (R5P3E) (EC 5.1.3.1), with the help of which several full-size cDNAs were isolated from spinach. Functional identity of the encoded mature subunit was shown by R5P3E activity found in affinity-purified glutatione *S*-transferase fusions expressed in *Escherichia coli* and by three-fold increase of R5P3E activity upon induction of *E. coli* overexpressing the spinach subunit under the control of the bacteriophage T_7 promoter, demonstrating that we have cloned the first functional ribulose-5-phosphate 3-epimerase from any eukaryotic source. The chloroplast enzyme from spinach shares about 50% amino acid identity with its homologues from the Calvin cycle operons of the autotrophic purple bacteria *Alcaligenes eutrophus* and *Rhodospirillum rubrum*. A R5P3E-related eubacterial gene family was identified which arose through ancient duplications in prokaryotic chromosomes, three R5P3E-related genes of yet unknown function have persisted to the present within the *E. coli* genome. A gene phylogeny reveals that spinach R5P3E is more similar to eubacterial homologues than to the yeast sequence, suggesting a eubacterial origin for this plant nuclear gene.

Abbreviations: R5P3E, D-ribulose-5-phosphate 3-epimerase; RPI, ribose-5-phosphate isomerase; TKL, transketolase; PRK, phosphoribulokinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBP, fructose-1,6-bisphophatase; FBP, fructose 1,6-bisphosphate; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; OPPP, oxidative pentose phosphate pathway; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; FBA, fructose-1,6-bisphophate aldolase; IPTG, isopropyl β-D-thiogalactoside; GST, glutathione *S*-tranferase; PBS, phosphate-buffered saline; TPI, triosephosphate isomerase

Introduction

Ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) catalyses the reversible interconversion of ribulose-5-phosphate and xylulose-5-phosphate. In animals and fungi, the 46 kDa homodimeric holoenzyme is integral to the classical F-type oxidative pentose phosphate pathway [18, 52], and may possess D-erythrose-4-phosphate isomerase activity [47]. Bacterial ribulose-5-phosphate 3-epimerase is encoded as a 25 kDa subunit in the Calvin cycle operons of *Alcaligenes eutrophus* [22] and *Rhodospirillum rubrum* [9]. The protein from human erythrocytes exhibits cooperativity between subunits [18], but otherwise only sparse kinetic data are available for the enzyme. Though a few eukaryotic expressed sequence tags (ESTs) and bacterial sequences exist in the database which possess similarity to *Alcaligenes* ribulose-5-phosphate 3-epimerase, no molecular clones from eukaryotes have been reported to date which have been demonstrated to encode the active, functional enzyme.

Even less is known about ribulose-5-phosphate 3-epimerase (R5P3E) of plants. This Calvin cycle enzyme has not yet been purified to homogeneity from any plant. Like ribose-5-phosphate isomerase (RPI, EC 5.3.1.6) and transketolase (TKL, EC 2.2.1.1), R5P3E is integral to both the Calvin cycle and oxidative pentose phosphate pathway, as illustrated in Fig. 1. It was recently shown that spinach leaves contain only a chloroplast-located enzyme for R5P3E, indicating that this chloroplast enzyme (in addition to RPI and TKL) is amphibolic, i.e. catalyses the corresponding reaction of both pathways in such tissues [37]. The results of that study further indicated that the enzymes of the classical (complete) oxidative pentose phosphate pathway are confined predominantly, if not exclusively, to the chloroplast, and that the cytosolic 'pathway' in spinach leaves (and perhaps other plant tissues) consists only of cytosolic isoenzymes for glucose-

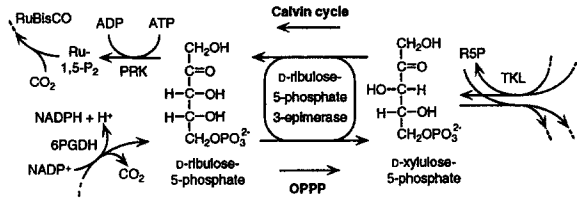

Fig. 1. Function of ribulose-5-phosphate 3-epimerase in plant metabolism. Abbreviations: PRK, phosphoribulokinase; 6GPDH, 6-phosphogluconate dehydrogenase; TKL, transketolase; R5P, ribose-5-phosphate; Ru-1,5-P_2, ribulose-1,5-bisphosphate; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase. Arrows indicate metabolite flux for the pathways indicated.

6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH). This is in contrast to the enzymes of glycolysis and gluconeogenesis, the activities of which exist as isoenzymes specific to the chloroplast and cytosol, respectively [7, 38].

With the exceptions of R5P3E, TKL and RPI, molecular probes for all nuclear-encoded enzymes of the Calvin cycle in chloroplasts of higher plants have been reported [2, 4, 15, 19, 24, 31–33, 36]. cDNA clones for several transketolases were also recently reported from rehydrating tissues of the resurrection plant *Craterostigma* [1] although it is not clear whether these cDNAs encode chloroplast (Calvin cycle) enzyme(s), due in part to their lack of transit peptide and to the unusual octulose metabolism in this totally dehydratable plant.

With molecular probes for all enzymes of the Calvin cycle one could study coordinated gene regulation in addition to enzyme activity for the complete set of enzymes from the pathway in various plants and tissues. The differential expression of Calvin cycle enzymes in the mesophyll and bundle-sheath of C4 plants [16, 20, 27, 44, 45] provides a convenient and effective tool for the isolation of molecular probes for Calvin cycle genes. Here we have used the differential

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number L42328.

screening approach with subtracted mesophyll and bundle-sheath cDNA probes to isolate the first plant cDNA for R5P3Es from bundle-sheath cells of the C4 plant *Sorghum bicolor*. That probe was used in turn to isolate a full-size clone from a cDNA library constructed from a tissue in which only one R5P3E enzyme is found. We show via expression of the predicted 25 kDa mature subunit in *E. coli* as glutathione *S*-tranferase fusion and phage T_7 promoter constructs that these cDNAs encode the enzymatically active R5P3E in spinach.

Materials and methods

Plant material

Sorghum bicolor cv. Tx430 (Pioneer Hi-Bred, Plainview, TX) was grown for 8 to 10 days in the greenhouse under the conditions described in Kubicki *et al.* [20].

Construction and differential screening of sorghum *cDNA library*

RNA was prepared from total leaves or isolated mesophyll protoplasts and bundle-sheath strands essentially as described [49]. A total leaf cDNA library was constructed in λZAPII using the cDNA cloning kit from Stratagene Cloning Systems (San Diego, CA). The amplified library was screened differentially [35] with single-stranded, radiolabelled cDNAs reverse-transcribed from mesophyll and bundle-sheath poly(A)$^+$ RNA. Putative positive phages were reconfirmed by rehybridization with the mesophyll and bundle-sheath probes and were sorted by cross-hybridization analysis as will be described in detail elsewhere [Wyrich *et al.*, manuscript in preparation].

Spinach cDNA cloning

For isolation of spinach clones, 10 ng of the cDNA previously synthesized [15] was ligated into 200 ng of *Eco* RI-digested λnm1149 [29] and packaged with commercial extracts (Stratagene). Recombinants were plated on *E. coli* POP13 and screened by plaque hybridization on 82 mm nitrocellulose filters (Gelman Sciences) at 55 °C in 3 × SSPE, 0.1% (w/v) SDS, 0.02% (w/v) PVP, 0.02% (w/v) Ficoll-400 and 50 μg/ml calf thymus DNA containing 20 ng/ml of hybridization probe. The hybridization probe was the 1 kb *Xho* I-*Eco* RI fragment of pHHU60 random-labelled to 3×10^7 cpm/μg [10] with α-^{32}P dCTP. Filters were washed for 60 min at 55 °C in 2 × SSPE, 0.1% (w/v) SDS and autoradiographed overnight on XAR films. Nested deletions for sequencing and other molecular methods were performed as described [15].

Construction of expressing strains

For expression as a glutathione *S*-transferase (GST) fusion, the coding region corresponding to the predicted mature subunit was ligated into *Xho* I-*Not* I-cut pGEX4T-2 (Pharmacia) as a 989 bp *Xho* I-*Not* I fragment from pRE91. The resulting construct (pRE91Δ^{53}gst) encodes an in-frame fusion of GST with the pRE91-encoded protein lacking 53 of the 58 amino-terminal residues corresponding to the transit peptide of pRE91 (see Results). Fusion sites of *amp*^{r} transformants in *E. coli* nm522 (Stratagene) were checked by sequencing with the primer 5′-GGCAAGCCACGTTTGGT-3′ from the GST-coding region. Affinity purified proteins (see below) from five independent 50 ml cultures bearing in-frame fusions were shown to exibit R5P3E acitivity, one of these was designated as strain nm522/pRE91Δ^{53}gst and was used in further analyses.

For expression under the control of the T_7 promoter [46], the region corresponding to the mature subunit was amplified under the conditions described [15] with the primers 5′-GTGTACATATGAAGGCAACATCTCGAGTTGACAAG-3′ and 5′-TTTAAGGATCCTTGGAAACTTCAGACTGCAACAG-3′ (start and stop codons in bold face), purified on Microcon 30 devices (Amicon), cut with *Bam* HI and *Nde* I and ligated into *Bam* HI/*Nde* I-cut pET-3a [46].

Amp^r transformants in *E. coli* BL21 were checked by restriction, one of these (BL21/pRE91$\Delta^{50}T_7$) was used in further analyses.

Expression in E. coli

nm522/pRE91Δ^{53}gst was grown to an OD_{600} of 0.6 at 37 °C in 500 ml of LB medium [35] containing 100 mg/l ampicillin and 2% glucose, induced by addition of 1 mM isopropyl β-D-thiogalactoside (IPTG), and allowed to grow for three further hours. Harvested cells from 1 litre of culture were resuspended with 20 ml PBS, lysed by sonification and incubated for 30 min on ice after addition of 1 ml of 20% (v/v) Triton X-100. The lysate was cleared by centrifugation for 10 min at 10 000 × *g* and 4 °C. Proteins from the supernatant were affinity-bound on a 500 µl glutathione-Sepharose column (Pharmacia) and washed with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) according to the manufacturer's protocol. Proteins were eluted with 2 ml of 5 mM glutathione, 50 mM Tris-HCl, pH 8.0, digested overnight at 4 °C with 8 U/ml thrombin (Serva) to remove the GST fusion and subsequently freed of glutathione via successive diafiltration with PBS in Minicon 10 devices (Amicon). The retentate was diluted to 2 ml with PBS and applied to a fresh glutathione-Sepharose column, the flow through was collected and concentrated in Minicon 10 devices. The nm522 wt control was handled in the same manner.

BL21/pRE91$\Delta^{50}T_7$ was grown to an OD_{600} of 1.7 at 37 °C in 200 ml of LB medium containing 20 mg/l ampicillin. Fifty ml of fresh medium were added. After 1 h cells were induced by addition of 0.4 mM IPTG and 1 ml samples taken at 1 h intervals. Cells were harvested, washed with lysis buffer (50 mM Tris-HCl, 2 mM EDTA, pH 8.0), recentrifuged and suspended in 400 µl lysis buffer containing 100 µg/ml lysozyme, incubated 15 min at 30 °C, vortexed and centrifuged. 10 µl of supernatant was used for SDS-PAGE, 30 µl for activity assay. The BL21 wt control was handled in the same manner.

Protein determination and SDS-PAGE

Protein was estimated by the method of Bradford [3] as described [35] with bovine serum albumin as a standard. For SDS-PAGE, samples from GST purification steps were desalted on 1 ml Sephadex-G25 columns. The eluate was precipitated with acetone and centrifuged. Dried pellets were dissolved in 20 µl of Laemmli buffer and boiled. Ten µl were loaded per lane. SDS-PAGE was performed as described [35].

Enzyme assay

R5P3E activity was assayed at 25 °C in 1 ml of 50 mM Tris, pH 7.4, 4.5 mM $MgCl_2$, 1 mM thiamine pyrophosphate, 1 U/ml transketolase (Sigma), 5.5 U/ml TPI (Boehringer), 0.5 U/ml glycerol-3-phosphate dehydrogenase (Boehringer), 240 µM NADH, 200 µM erythrose-4-phosphate, and 100 µM ribulose-5-phosphate. The commercially available yeast enzyme (Sigma) was used as a control. One unit is the amount of enzyme required to convert 1 µmol of substrate per min.

Genomic Southern blots

Twenty µg of spinach DNA was digested with the appropriate enzyme, electrophoresed, blotted and hybridized as described [15]. Three hybridization probes derived from pRE91 were used: the 989 bp *Xho* I-*Not* I fragment (coding region of the mature subunit), the 218 bp *Xho* I-*Not* I fragment (transit peptide and 5′ end) and the 364 bp *Not* I-*Pst* I fragment (3′ end). Probes were random-labelled [10] to ca. 10^8 cpm/µg with α-^{32}P dCTP, added to the hybridization solution to a final concentration of 10 ng/µl and hybridized to 3 × $c_o t_{1/2}$. Filters were washed for 60 min at 68 °C in 2 × SSPE, 0.1% (w/v) SDS and autoradiographed for three days on XAR films.

Sequence analysis

Standard sequence processing was performed with the GCG Package [8]. Sequence alignments were created with CLUSTAL V [17] and refined by eye. Distance between sequences for phylogenetic inference with the neighbour-joining method [34] was measured as numbers of amino acid substitutions per site corrected for multiple substitutions with the Dayhoff matrix of PHYLIP [11].

Results

Isolation and sequence of R5P3E clones

From the differential screening of the *Sorghum* cDNA library with mesophyll and bundle-sheath-specific hybridization probes we obtained 62 different cDNA clones which were characterized by partial sequencing and RNA expression analysis (Wyrich *et al.*, manuscript in preparation). The encoded protein of one of these, pHHU60 (accession number H55034), revealed 52% identity over 94 amino acids to *Alcaligenes* and *Rhodospirillum* R5P3E proteins. From 100 000 recombinants of the unamplified spinach cDNA library screened with the pHHU60 insert we obtained independent 20 positively hybridizing cDNAs. Nine of these were purified and contained hybridizing inserts of 1 to 1.2 kb in length. Five of these were subcloned as *Not* I fragments into pBluescript vectors (Stratagene) and shown by sequencing to be identical in sequence over >200 bases of their 5′ and/or 3′ ends to pRE91 (Fig. 2). Several of these contained an 858 bp open reading frame with similarity to R5P3E. pRE13 possess a good consensus polyadenylation signal (ATAAAA) 21 bp upstream of the polyadenylation site. pRE91 possesses an $A_{(60)}$ tract at its 5′ end which could be a cloning artefact since only found in one clone, but two independent cDNA clones for spinach Calvin cycle TPI [15] possess a $T_{(30)}$ tract at identical positions in their 5′ ends. The significance of these homopolymer tracts is not obvious, but their occurrence seems notewor-

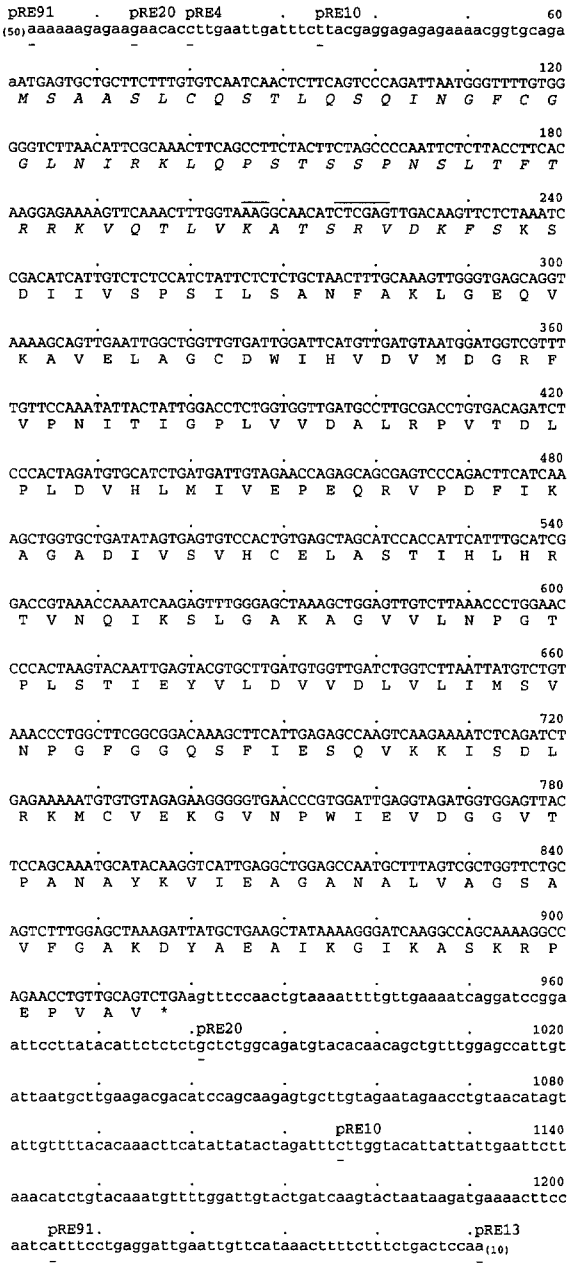

Fig. 2. Sequence of cDNA clones for chloroplast ribulose-5-phosphate 3-epimerase from spinach. Terminal nucleotides of clones pRE4, pRE10, pRE13, pRE20 and pRE91 (underlined) are indicated (accession number L42328). The expected transit peptide is shown in italics. The *Xho* I site used for construction of GST fusion and the lysine codon following the start codon for expression in the T_7 system [46] are overlined. Non-coding regions are shown in lower case. The sequence of pHHU60 determined corresponds to positions 375–700 in the spinach sequence.

thy. The region of similarity in the encoded spinach protein to other sequences (see below) is preceeded by a 58 amino acid serine-rich amino-terminal extension with typical properties of chloroplast transit peptides [48]. Since the processing site of precursors for other nuclear-encoded chloroplast proteins is known to lie close to the amino-terminus of the bacterial or eukaryotic homologues of the cytosol in corresponding alignments [28, 51], the alignment of the pRE91 encoded protein (Fig. 3) suggests a processing site close to that indicated in Fig. 2, the predicted transit peptide is 58 amino acids in length. The size of the predicted 227 amino acid mature subunit is 24.1 kDa, similar to that of R5P3E from *Alcaligenes* [22]. The spinach protein is 90% identical over 97 residues to the deduced partial amino acid sequence of pHHU60 from *Sorghum* (corresponds to nucleotide positions 375–700 in Fig. 2, data not shown). This degree of conservation between monocot and dicot sequences is typical of enzymes of sugar phosphate metabolism.

R5P3E-related proteins

We retrieved complete sequences from the database which are related to the pRE91-encoded protein. The pRE91 encoded reading frame showed the highest amino acid identity to the biochemically characterized R5P3E from *Alcaligenes eutrophus* [22] and the *dod* gene product of *Serratia marcescens* (X78412) (50% identity each), the lowest among significant scores ($P < 10^{-6}$) was found to an *E. coli* R5P3E-related protein (P32719, 32% identity). An alignment of the pRE91 encoded protein to related sequences is shown in Fig. 3.

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                                                                                                  90
Spinach Chloroplast              <<KSDIIVSPSILSANFAKLGEQVKAVELAGCDWIHVDVMDGRFVPNITIGPLVVDALRPVTD LPLDVHLMIVEPEQRV
Alcaligenes eutrophus  MHATELNTGHGSQRAIRLAPSILSADFARLGEEVCAIEAGGADLVHFDVMDNHYVPNLTIGPLVCEAIRPLVS·IPIDVHLMVEPVDALI
Rhodospirillum rubrum            MTRAIRIAPSLLSADFAISRPRCPSDGRTGADILHFDVMDNHYVPNLTVGPLVCAALRPHTS·LPIDVHLMTRPVDPLI
Serratia marcescens               MKKFLIAPSILSADFARLGEDTANVLAAGGDVVHFDVMDNHYVPNLTIGPMVCEALRNYGITAPIDVHLMVKPVDRIV
Escherichia coli 1                MKQYLIAPSILSADFARLGEDTAKALAAGADVVHFDVMDNHYVPNLTIGPMVLKSLRNYGITAPIDVHLMVKPVDRIV
Escherichia coli 2                   MKISPSLMCMDLLKFKEQIEFIDSH·ADYFHIDIMDGHFVPNLTLSPFFVSQVKKLAT·KPLDCHLMVTRPQDYI
Escherichia coli 3                   MILHPSLASANPLHYGRELTALDNLDFGSLHLDIEDSSFINNITFGMKTVQAVARQTP·HPLSFHFMLARPQRWF
Saccharomyces                     MVKPIIAPSILASDFANLGCECHKVINAGADWLHIDVMDGHFVPNITLGQPIVTSLRRSVPTAFFDCHMMVENPEKWV
                                        **                                * * *    * *                  |          * *
                                                                                                  RPGDASNTEKKP

                                                                                                 180
Spinach Chloroplast    PDFIKAGADIVSVHCELASTIHLHRTVNQIKSLGAKAGVVLNPGTPLSTIEYVLDVVDLVLIMSVNPGFGGQSFIESQVKKISDLR····
Alcaligenes eutrophus  PLFAKAGANIISFHPE··ASRHVDRTIGLIRDHGCKAGLVLNPATPLGWLDHTLDQLDLVLLMSVNPGFGGQAFIPGVLDKVRQARARID
Rhodospirillum rubrum  DSFAEAGADMITFHPE··ASDHVHRSVQMIRKRGLKAGVALNPASPLSLLDHILEDLDLVLIMSVNPGFGGQSFIPSALPKIAALA····
Serratia marcescens    PDFAKAGASYISFHPE··ASEHVDRTIQLIKEHGCKAGLVFNPATPLSYLDYVMDKIDVILLMSVNPGFGGQSFIHGTLDKLRQVR····
Escherichia coli 1     PDFAAAGASIITFHPE··ASEHVDRTLQLIKENGCKAGLVFNPATPLSYLDYVMDKLDVILLMSVNPGFGGQSFIPQTLDKLREVR····
Escherichia coli 2     AQLARAGADFITLHPE·TINGQAFRLIDEIRRHDMKVGLILNPETPVEAMKYYIHKADKITVMTVDPGFAGQPFIPEMLDKLAELK····
Escherichia coli 3     NALAEIRPAWIFVHAE··TLDYPSETLTEIRHTGARAGLVFNPATPIDAWRYLASELDGVMVMTSEPDGQGQRFIPSMCEKIQKVR····
Saccharomyces          DDFAKCGADQFTFHYE··ATQDPLHLVKLIKSKGIKAACAIKPGTSVDVLFELAPHLDMALVMTVEPGFGGQKFMEDMMPKVETLR····
                             * *                          *                    *   *   ** *      *

                                                    250
Spinach Chloroplast    KMCVEKGVNPWIEVDGGVTPANAYKVIEAGANALVAGSAVFGAKDYAEAIKGIKASKRPEPVAV*
Alcaligenes eutrophus  RQVDAGGRPVWLEIDGGVKADNIAAIARAGADTFVAGSAVFGAPDADGGYSSILYRLREAATVT*
Rhodospirillum rubrum  ··NGRRRACRGDRVDGGVNPADARALARPGADILVRL·AIFGASDRAKAIASIRGAAESGLGQEAA*
Serratia marcescens    KLIDDSGRDIRLEVDGGVKVDNIAEIAAAGADMFVAGSAIFGQPDYRK*
Escherichia coli 1     RRIDESGFDIRLEVDGGVKVNNIGEIAAAGADMFVAGSAILTSQTTKKSLMKCAVNWQR*
Escherichia coli 2     AWREREGLEYEIEVDGSCNQATYEKLMAAGADVFIVGTSGLFNHAENIDEAWRIMTAQILAAKSEVQPHAKTA*
Escherichia coli 3     ····TAFPQTECWADGGITLAAAQQLAAAGAQHMVIGRALFSSSDYRATLAQFATL*
Saccharomyces          ····AKFPHLNIQVDGGLGKETIPKAAKAGANVIVAGTSVFTAADPHDVISFMKEEVSKELRSRDLLD*
                                  ***          /*
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Fig. 3. Alignment of ribulose-5-phosphate 3-epimerase related protein sequences. Sources of sequences are: *E. coli*1, Z19601; *E. coli*2, P32719; *E. coli*3, P39362; *Alcaligenes*, M64173; *Rhodobacter*, S64484; *Serratia*, X78412; yeast, X83571. Strictly conserved residues are indicated with an asterisk. The *Rhodospirillum rubrum* sequence shown here was amended from the database accession by insertion of an additional guanosine in the conserved codon G_{210} of the figure (indicated with '/'), which markedly improved the subsequent alignment of that sequence. The dodecapeptide below the yeast sequence has been set apart from the alignment since the corresponding DNA insertion relative to the other sequences (5′-gtgccacgccctggcgatgctagcaacacagaaaag-3′) is suspiciously intron-like and since the size of the shortened predicted protein (24.7 kDa) is closer to that previously reported for the enzyme (23 kDa [50]).

Identification of enzyme function

In order to determine whether pRE91 encodes the R5P3E activity detected in spinach leaves [37], we utilized the convenient *Xho* I site indicated in Fig. 2 to generate the construct pRE91Δ^{53}gst, in which the mature subunit and five amino acids of the predicted transit peptide are expressed as an in-frame GST fusion. Proteins from *E. coli* strain nm522/pRE91Δ^{53}gst purified by affinity chromatography on glutathione-Sepharose revealed only two major bands (lane 2, Fig. 4). The 51 kDa protein corresponds to the expected size of the pRE91Δ^{53}gst-encoded product. A roughly 24 kDa band was sometimes observed as a series of 30–24 kDa bands (data not shown) and probably corresponds to degradation products of the fusion protein. A weak 60 kDa band of unknown function was observed in all steps (prominent in lane 4, Fig. 4) which also purifies from *E. coli* wild-type (wt) strains on glutathione-Sepharose but has no detectable enzyme activity (data not shown). The thrombin cleavage products of the pRE91Δ^{53}gst fusion have a size of 27.5 kDa (GST) and 25 kDa (pRE91Δ^{53}), and are not separated effectively under our electrophoresis conditions (Fig. 4, lane 3). When the products of thrombin cleavage are reapplied to glutathione-Sepharose, only two bands are visible, and a small quantity of 25 kDa protein is found in the flow-through (Fig. 4, lane 4).

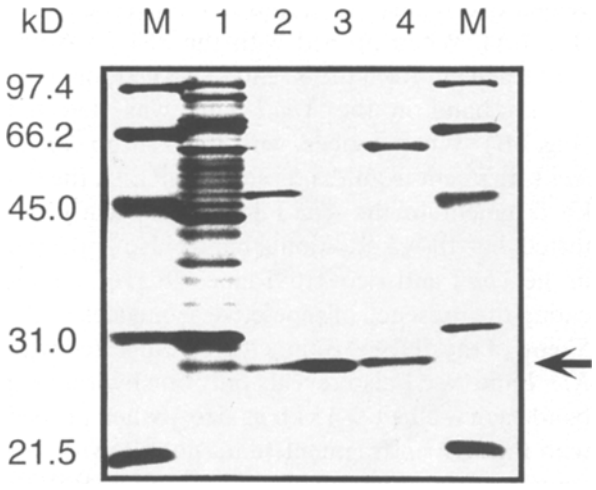

Fig. 4. Coomassie-stained SDS-PAGE of fractions from affinity purification of proteins from nm522/pRE91Δ^{53}gst. Lane 1, crude extract; lane 2, glutathione eluate; lane 3, eluate after thrombin digestion; lane 4, thrombin-digested eluate rechromatographed on glutathione-Sepharose (flow-through). An arrow indicates the 25 kDa band corresponding to the thrombin-released epimerase protein. Sizes of molecular mass standards (kDa) are indicated.

Enzyme activity was monitored during affinity purification. The overall level of expression in *E. coli* with the glutathion fusion was low, and degradation of the fusion product was a problem. The fusion protein band was not prominent enough to be identified in SDS-PAGE of total proteins from nm522/pRE91Δ^{53}gst during induction kinetics or in comparison to *E. coli* nm522 wt (data not shown). In addition, thrombin cleavage was difficult with this protein. Use of sufficient thrombin to cleave the 51 kDa fusion protein to completion also led to reduction of epimerase activity. Cleavage at 25 °C also resulted in loss of epimerase activity. As shown in Table 1 and Fig. 4, GST affinity chromatography efficiently removed total protein but the increase in specific activity was very low except for the final flow-through. Nonetheless, epimerase activity could be specifically eluted as GST-fusion protein with glutathione. Subsequent to thrombin cleavage and

Table 1. Epimerase activity during affinity chromatography on glutathion Sepharose of proteins extracted from *E. coli* nm522 harbouring and lacking pRE91Δ^{53}gst. Total proteins (crude extract) were applied to glutathion Sepharose. The column was washed (final wash), proteins were eluted with 5 mM glutathion (glutathione eluate) and digested to release GST from the fusion protein (thrombin-cleaved). Cleaved proteins were reapplied to glutathione Sepharose in order to remove GST, the flow-through was collected (final flow-through).

Sample	Specific activity (U/mg)	
	nm522	nm522/pRE91Δ^{53}gst
Crude extract	0.02	0.02
Final wash	0	0
Glutathione eluate	0	0.09
Thrombin-cleaved	0	0.05
Final flow-through	0	0.28

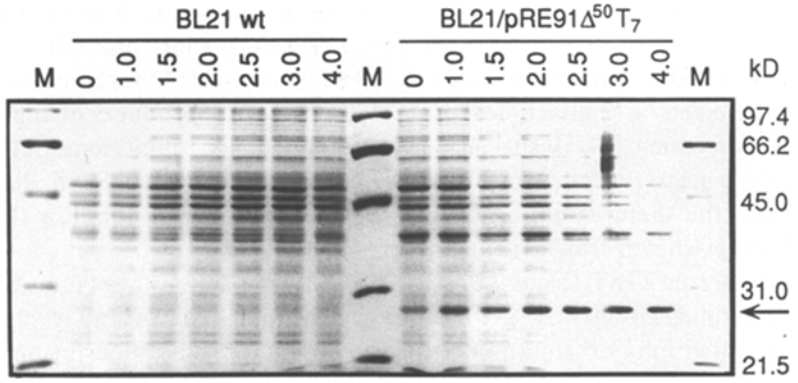

Fig. 5. Coomassie-stained SDS-PAGE of induction kinetics of R5P3E in BL21/pRE91Δ^{50}T$_7$. Aliquots were taken from the culture at times after induction (h) indicated. The arrow indicates the induced 26 kDa protein. Sizes of molecular weight standards (kDa) are indicated.

removal of glutathione, epimerase activity did not bind, but rather accumulated in the flow-through as expected.

Expression in the T_7 system yielded much higher total activity and a more stable product. The induction kinetics of BL21/pRE91Δ^{50}T$_7$-encoded epimerase is shown in Fig. 5. The specifically induced ca. 25 kDa band has the expected molecular mass for spinach epimerase and is the major soluble protein in cells harboring the plasmid. Total R5P3E activity in BL21/pRE91Δ^{50}T$_7$ overexpressing cells is twice that of the BL21 wt control prior to induction, increasing to three fold wt levels after 4 h (Table 2).

Table 2. R5P3E activity in crude extracts of *E. coli* BL21 strains harboring and lacking pRE91Δ^{50}T$_7$. Values in parenthesis indicate activity found when IPTG induction was performed at an OD_{600} of 2.2 rather than 1.7.

Time after induction (h)	Crude extract R5P3E activity (U/ml)	
	BL21	BL21/pRE91Δ^{50}T$_7$
0	0.022 (0.042)	0.047 (0.158)
1.0	0.025 (0.042)	0.052 (0.153)
1.5	0.022 (0.042)	0.090 (0.150)
2.0	0.024 (0.037)	0.058 (0.121)
2.5	0.027 (0.037)	0.126 (0.110)
3.0	0.031 (0.042)	0.131 (0.134)
4.0	0.029 (0.037)	0.120 (0.150)

R5P3E nuclear genes in spinach

When total spinach DNA was digested with enzymes which do not cut in the pRE91 cDNA sequence and probed under low-stringency conditions with the 989 bp *Xho* I-*Not* I fragment from pRE91, only one hybridizing band was detected in EcoO109I (6.8 kb) digests, whereas *Xba* I revealed two bands of 7.2 and 0.9 kb, respectively (Fig. 6A). When probed with the 364 bp *Not* I-*Pst* I fragment from the 3′ end of pRE91, only the 7.2 kb band in the *Xba* I lane was detected (Fig. 6B). When probed with the 218 bp *Xho* I-*Not* I fragment from the 5′ end of pRE91, the 0.9 kb fragment in the *Xba* I lane was clearly detected, but 10–15 additional bands also appeared in the *Xba* I and EcoO109I lanes (Fig. 6C), indicating the presence of repetetive sequences in the 5′ end of the cDNA. Spinach DNA digested with *Kpn* I and *Sac* I also reveals only one hybridizing band each – albeit > 15 kb in size – when probed with the 989 bp fragment (data not shown). We cannot exclude the possibility that other R5P3E proteins are encoded in spinach DNA by genes which are not detected by low-stringency hybridization, but the results of the Southern blots, taken together, indicate that the coding region of R5P3E occurs as a single copy in the spinach genome by a gene which contains an intron with an *Xba* I site.

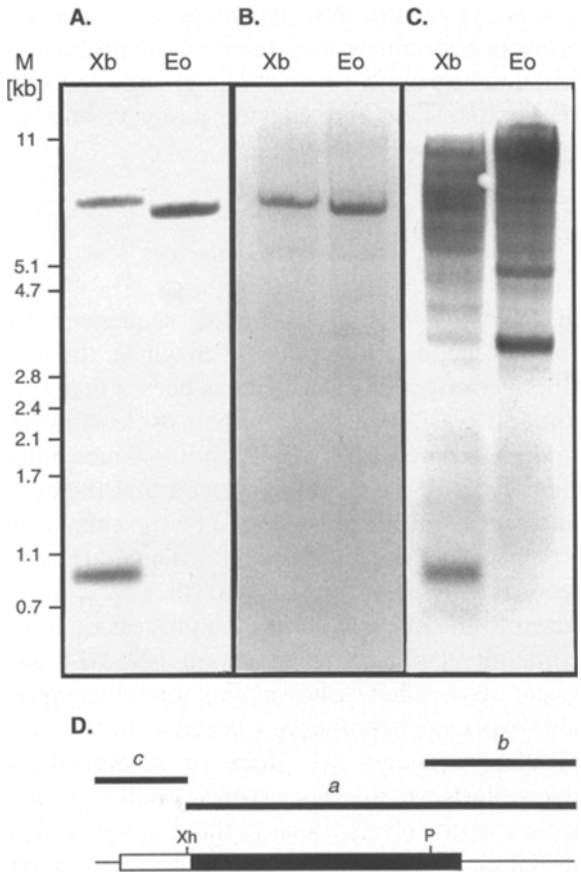

Fig. 6. Southern blot of genomic DNA from spinach probed fragments of pRE91. 20 μg of spinach DNA was loaded per lane. Eo, EcoO109I; M, marker; P, *Pst* I; Xb, *Xba* I; Xh, *Xho* I. Sizes of molecular weight standards (kb) are indicated. A. Blot probed with the 989 *Xho* I-*Not* I fragment encoding the mature subunit of R5P3E. B. Blot probed with the 364 bp *Not* I-*Pst* I fragment (3′ end). C. Blot probed with the 218 bp *Xho* I-*Not* I fragment (5′ end). D. Schematic representation of the pRE91 cDNA. Open box: transit peptide coding region; solid box: region encoding the mature subunit; thin lines: non-coding regions; bold lines: probes used in panels A, B and C, respectively. Films were exposed for 24 h at −80 °C.

Discussion

Results of overexpression experiments in *E. coli* conclusively demonstrate that we have cloned cDNAs for functional R5P3E from spinach. cDNA cloning revealed an abundance for this transcript of roughly 1/5000 of poly(A)$^+$ mRNA, R5P3E mRNA is thus about five-fold less abundant than that for the A subunit of Calvin cycle GAPDH in most green tissues [28] but about two-fold higher than that for Calvin cycle TPI in the same tissue [15]. As revealed by cell fractionation studies and ion-exchange chromatography, spinach leaves appear to possess only a single, chloroplast-located R5P3E enzyme [37]. This is consistent with our findings that the region of pRE91 encoding the active spinach enzyme is preceeded by a transit peptide-like region of roughly 58 amino acids and that only one class of transcript was detected. The degree of conservation between the spinach and sorghum sequences, in addition to the differential expression pattern of the sorghum clone suggests that it also encodes a functional enzyme.

The two other enzymes shared by the Calvin cycle and OPPP in this spinach leaves, RPI and TKL, appear also to occur as single, chloroplast-located enzyme species [37]. With the notable exception of the first two dehydrogenases [13, 30, 39, 40, 42, 43], enzymes of the OPPP in plants have been neglected at both the biochemical and molecular level. Work is currently in progress to study these in more detail and to isolate clones for these enzymes in order to determine whether amphibolic Calvin cycle/OPPP enzymes may share coordinated patterns of gene expression as they do in autotrophic bacteria, where the genes for Calvin cycle enzymes are coordinately regulated as operons under the control of positve regulators [6, 9, 12, 22].

R5P3E enzymes

The R5P3E sequence reported here from spinach chloroplasts is the first for this enzyme from any eukaryote to which a function can be assigned. The active subunit appears to have a similar size (ca. 24 kDa) as the enzyme from bacteria (25.5 kDa [22]), vertebrates (23 kDa [18]) and yeast (23 kDa [50]). Activity detected in the overexpressed and affinity-purified GST-fusion protein isolated from *E. coli* suggest that the spinach enzyme does not require additional subunits for activity. A R5P3E-related sequence was recently reported from yeast (X83571), the predicted sub-

unit size of which is slightly higher (26 kDa) than that for the native enzyme, and which may contain an intron not reported in the accession (Fig. 3); the function of the R5P3E-related sequence from yeast is not known. Also, the extremely GC-rich *Rhodospirillum rubrum* sequence probably has a frame shift (five cytosines instead of four at postions 154–157 in S64484) since the amended sequence is easily aligned with its functional homologues from spinach chloroplast and *Alcaligenes*.

The function of the *Alcaligenes* gene product was demonstrated by overexpression and activity assay, as reported here. The function of the remaining sequences has not been determined, although there is little doubt that the *cbbE* gene product of *R. rubrum* [9] is functional, since it is located in an active Calvin cycle operon in that bacterium and since the operon structure of that Type II (L_4) Rubisco-encoding Calvin cycle gene cluster (*RPE*-FBP-PRK-TKL) is conserved with that of the *A. eutrophus* chromosomal Calvin cycle operon, which however encodes Type I (L_8S_8) Rubisco (*rbcL-rbcS*-cfxX-cfxY-*RPE*-FBA-PRK-TKL-GAPDH) [22]. Interestingly, neither form I (FBP-PRK-FBA-*rbcL-rbcS*) nor form II (L_2 Rubisco) (FBP-PRK-TKL-GAPDH-FBA-*rbcL*) Calvin cycle operons of *Rhodobacter sphaeroides* [12] encode R5P3I. A database search revealed three independent *E. coli* sequences with more than 30% amino acid identity to spinach R5P3E (Fig. 3), designated here for convenience as *E. coli*1, *E. coli*2 and *E. coli*3. *E. coli*1 (Z19601) is located behind the *dam* gene encoding desoxyadenosine methylase, as is the R5P3E-related homologue from *Serratia*. *E. coli*2 (P32719 and u00006) is situated between presumably co-transcribed genes with similarity to the *Bacillus subtilis* xylose repressor and *E. coli* RbsC high affinity ribose importer and thus may have some function in metabolism of (perhaps unphosphorylated) sugars. Neighboring sequences of *E. coli*3 (P39362) provide no hints at all regarding possible function. Several expressed sequence tags (ESTs) from rice (e.g. D47269) and *Arabidopsis* (e.g. D45235) were also found in an EST database search with similarity to spinach chloroplast R5P3E (L42328). As sequences from genome projects accumulate in databases, the problem of function assignment is becoming increasingly severe, expression and enzyme assay is one approach for metabolic proteins.

Evolution of R5P3E-related sequences

In previous investigations of sequences for nuclear-encoded Calvin cycle enzymes, the evolutionary origin of the genes has been a matter of some interest. For those Calvin cycle enzymes studied to date which possess homologues in the plant cytosol, it was long assumed that the cytosolic isoenzymes were encoded by the eukaryotic host nucleus prior to endosymbiosis and that the genes for the chloroplast isoenzymes were transferred to the nucleus during the process of endosymbiont genome size reduction [24, 41]. But recent studies have shown that this albeit plausible working hypothesis – known as the gene transfer corollary – does not apply for chloroplast/cytosol isoenzymes of the Calvin cycle and glycolysis, since in those cases studied in detail, genes for chloroplast/cytosol isoenzymes have been shown to be related through gene duplication, either in eubacterial (GAPDH [23]) or in eukaryotic (aldolase [31]; TPI [15]; PGK [5]) chromosomes. In the case of chloroplast/cytosol isoenzymes of the Calvin cycle and OPPP (as opposed to Calvin cycle glycolysis), the evolutionary origin of compartment-specific isoenzymes has been less heavily debated than has the question of whether such isoenzymes actually exist in plants [37]. Notwithstanding the fact that chloroplast R5P3E possesses no detectable homologue in the cytosol of spinach leaves [37], it was of interest to examine the gene phylogeny of the few R5P3E-related sequences currently available (Fig. 7). Although the gene tree bears only few branches, two observations are noteworthy. Firstly, it is quite clear that the duplication events which gave rise to the three *E. coli* genes (the encoded proteins of which share only 30% identity) took place long prior to the diversification of purple bacteria. Ancient gene dupli-

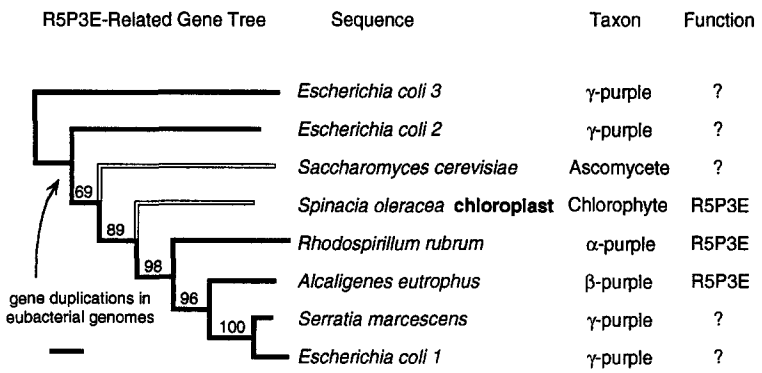

Fig. 7. Phylogenetic tree of ribulose-5-phosphate 3-epimerase related proteins. Sequences encoded in eubacterial genomes are shown on solid branches, those encoded in eukaryotic genomes are indicated on open branches. The tree was constructed using the Dayhoff matrix and neighbor joining options of PHYLIP, the branch indicates 0.1 substitutions per site. Question marks indicate that experimental evidence for function of the protein has not been reported. Numbers above branches indicate the number of times out of 100 Dayhoff distance-NJ tree bootstrap replicates that the branch was found. Database sources of sequences are given in the legend to Fig. 3.

cations of this type are also known to have occurred for other genes for enzymes of primary metabolism such as GAPDH [14], Rubisco [25, 26] and glutamine synthase [21]. Secondly, spinach chloroplast R5P3E is more similar to Calvin cycle homologues of eubacteria than to the R5P3E-like sequence from yeast. Under the most straightforward interpretation, this finding indicates a eubacterial (perhaps cyanobacterial) origin via endosymbiotic gene transfer of the nuclear spinach gene for chloroplast R5P3E, similar to the scenario observed for chloroplast GAPDH [14, 23] and chloroplast PGK [5]. A much more complicated alternative scenario involving additional gene duplications subsequent to those which gave rise to the three *E. coli* genes appears unlikely, but currently cannot be ruled out. From an evolutionary standpoint it will be of interest to study R5P3E genes from other photosynthetic eukaryotes and from cyanobacteria.

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