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Biosynthesis of riboflavin in plants. The *ribA* gene of *Arabidopsis thaliana* specifies a bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase

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Abstract

A cDNA segment from *Arabidopsis thaliana* with similarity to the *ribA* gene of *Bacillus subtilis* was sequenced. A similar gene was cloned from tomato. The open reading frame of *A. thaliana* was fused to the *malE* gene of *Escherichia coli* and was expressed in a recombinant *E. coli* strain. The recombinant fusion protein was purified and shown to have GTP cyclohydrolase II activity as well as 3,4-dihydroxy-2-butanone 4-phosphate synthase activity. The cognate gene was amplified by polymerase chain reaction from chromosomal *Arabidopsis* DNA and was shown to contain six introns. Intron 4 is located in the region connecting the GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase domain of the putative domains catalyzing the two reaction steps. By comparison with the bacterial *ribA* gene, the *Arabidopsis* gene contains an additional 5′ element specifying about 120 amino acid residues. This segment contains numerous serine and threonine residues and does not show similarity with other known sequences. The N-terminal segment is not required for catalytic activity and is likely to serve as signal sequence for import into chloroplasts.

Keywords: *Arabidopsis thaliana*; *Lycopersicon esculentum*; Brassicaceae; Solanaceae; Riboflavin biosynthesis; GTP cyclohydrolase II; 3,4-dihydroxy-2-butanon 4-phosphate synthase

1. Introduction

Vitamin B_2 (riboflavin) is biosynthesized in plants and in many microorganisms. The biosynthetic pathway (Fig. 1) has been studied in considerable detail in bacteria and yeasts (for review see Bacher, 1991; Bacher, Eberhardt & Richter, 1996). The biosynthetic formation of one molecule of riboflavin requires one molecule of GTP and two molecules of ribulose 5-phosphate. GTP (**1**) is initially converted to the committed product, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate (**2**) by the enzyme, GTP cyclohydrolase II. The enzyme product is converted to 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione (**3**) by a sequence of deamination, side chain reduction, and dephosphorylation. The pyrimidine **3** is converted to 6,7-dimethyl-8-ribityllumazine (**4**) by condensation with 3,4-dihydroxy-2-butanone 4-phosphate (**5**) which is obtained enzymatically from ribulose 5-phosphate (**6**) by 3,4-dihydroxy-2-butanone-4-phosphate synthase. The enzymatic formation of **5** from ribulose 5-phosphate involves an unusual skeletal rearrangement.

The *ribA* gene of *Bacillus subtilis* specifies a bifunctional protein catalyzing the formation of the pyrimidine **2** and of the carbohydrate **5** from GTP and ribulose-5-phosphate, respectively (Richter et al., 1993). Similar proteins have been predicted on the basis of DNA sequence information in several other microorganisms such as, *Bacillus amyloliquefaciens, Synechocystis sp.*, and *Mycobacterium tuberculosis* (Gusarov et al., 1997; Kaneko et al., 1996; Philipp et

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al., 1996). In other microorganisms such as, *Escherichia coli* and *Saccharomyces cerevisiae*, GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase are specified by unlinked genes (Richter, Volk, Krieger, Lahm, Roethlisberger & Bacher, 1992; Richter et al., 1993; Oltmanns, Bacher & Lingens, 1969; Oltmanns & Bacher, 1972).

The terminal enzyme of the biosynthetic pathway, riboflavin synthase, has been partially purified from spinach (Mitsuda, Kawai & Suzuki, 1976). Recently, 6,7-dimethyl-8-ribityllumazine synthase from spinach, tobacco and *Arabidopsis thaliana* has been cloned and characterized (Jordan, Bacot, Carlson, Kessel & Viitanen, 1999). No other enzyme of the riboflavin pathway has been characterized in plants.

We describe a gene from *Arabidopsis thaliana* which specifies a bifunctional enzyme catalyzing the initial steps of both branches of the convergent riboflavin biosynthesis pathway. The protein is closely similar to the bifunctional RibA protein of *B. subtilis*.

2. Results and discussion

2.1. Sequence analysis of the EST-clone 41G4T7

Sequence comparison showed that the EST clone 41G4T7 from *A. thaliana* predicts a protein with similarity to the 3,4-dihydroxy-2-butanone 4-phosphate synthase domain of the RibA protein of *B. subtilis*. This EST clone had been (incorrectly) annotated as riboflavin synthase (Newman et al., 1994).

The entire insert of EST clone 41G4T7 was sequenced by the automated dideoxynucleotide method using a primer walk strategy. The insert (designated i41G4T7) had a length of 2525 bp. Part of the resulting sequence (bp 1–398) had been published earlier in the EST database. The segment comprising bp 109–1297 is similar to the *ribA* gene of *B. subtilis*; the segments containing bp 563–1485 and 2262–2505 are identical with the *A. thaliana* Genbank sequence D45165 which was thought to be a GTP cyclohydro-

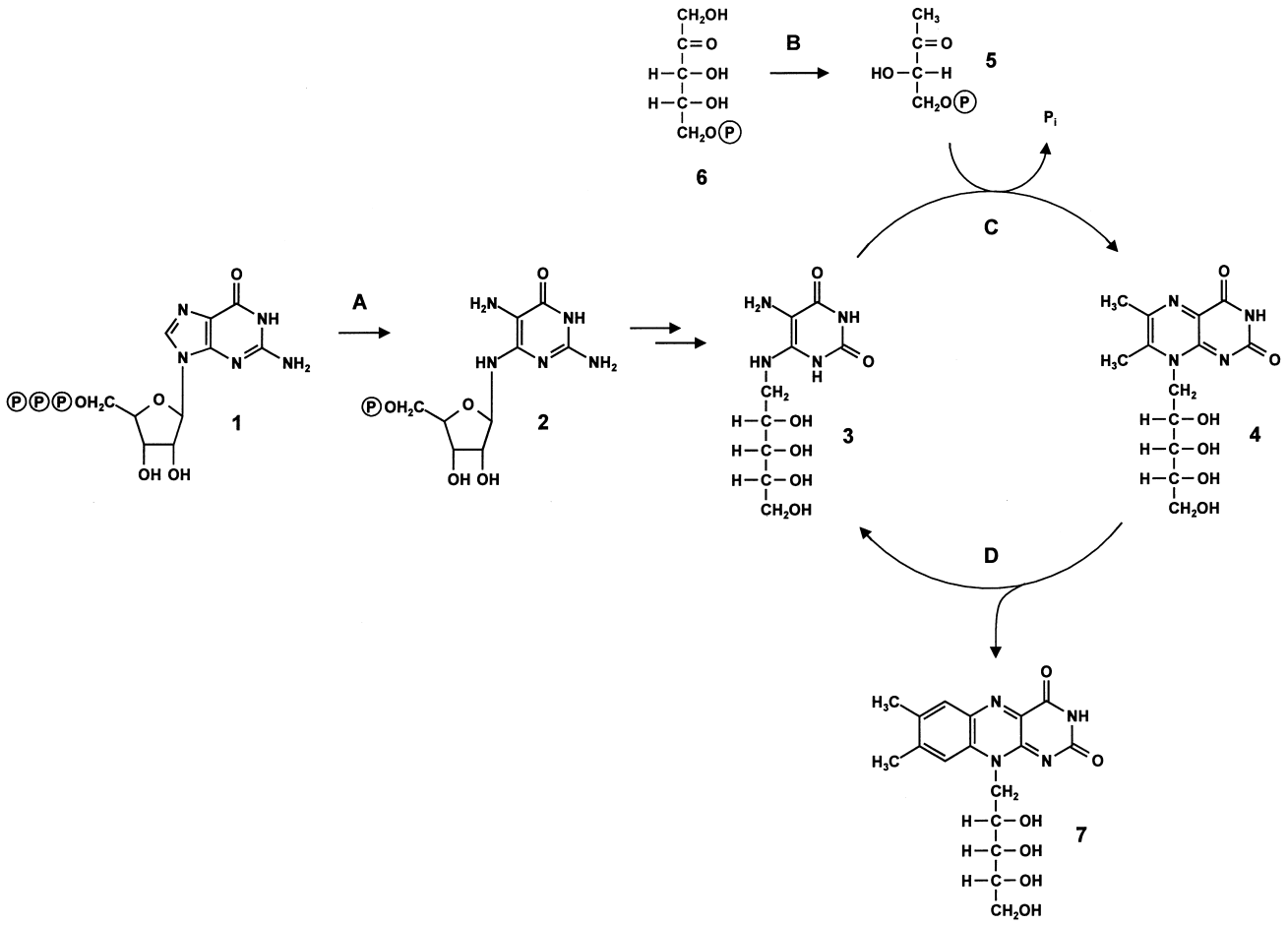

Fig. 1. Biosynthesis of riboflavin. **1**, GTP; **2**, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate; **3**, 5-amino-6-ribitylamino-2,4(1H, 3H)-pyrimidinedione; **4**, 6,7-dimethyl-8-ribityllumazine; **5**, 3,4-dihydroxy-2-butanone 4-phosphate; **6**, ribulose 5-phosphate; **7**, riboflavin; **A**, GTP cyclohydrolase II; **B**, 3,4-dihydroxy-2-butanone 4-phosphate synthase; **C**, lumazine synthase; **D**, riboflavin synthase.

lase II (Kobayashi, Sugiyama & Yamamoto, 1995). The transposon IS1 was located at bp 1486–2261.

2.2. DNA sequence extension

Comparison of the *Arabidopsis* sequence with the *B. subtilis ribA* gene suggested tentatively that the EST clone 41G4T7 did not contain the entire 5′ part of the gene under study. In order to determine the size of full length transcripts, we performed a Northern blot with mRNA from *Arabidopsis thaliana* var. Columbia using a hybridization probe obtained by PCR amplification of bp 39–1484 from clone 41G4T7. This probe contained parts of the putative 3,4-dihydroxy-2-butanone 4-phosphate synthase domain as well as the putative GTP cyclohydrolase II domain. We found a single band of about 2300 bp (Fig. 2). This suggested that the cDNA clone 41G4T7 was missing about 560 bp at the 5′ end.

In order to extend the known sequence in the 5′ direction, a RACE experiment was performed by reverse PCR using mRNA from *A. thaliana* as template. The RACE product, which had a length of 800 bp, was sequenced and was shown to contain 556 bp which were not present in clone 41G4T7. The coding region of the extended cDNA contains 1629 bp specifying a predicted protein with 543 aa and a mass of 59,055 Da. The cognate gene is subsequently designated *ribA*. The sequence around the first ATG triplet (**TAGAAAATGTCT**) shows similarity to the consensus sequence (TAAACAATGGCT) around plant initiation codons (Joshi, 1987). The 5′ untranslated region is 284 bp long. The 3′ untranslated region has a length of 372 bp and contains a typical polyadenylation signal (AATAAA) 17 bp upstream of the poly(A) tail (Proudfoot & Brownlee, 1976).

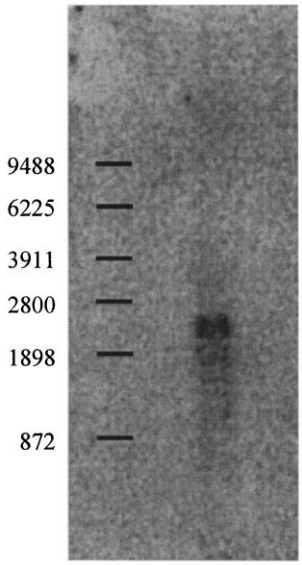

Fig. 2. Northern blot of *A. thaliana* mRNA. The blot was hydridized with *ribA*. For details, see methods.

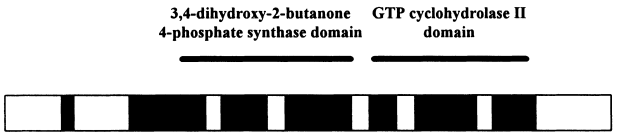

Fig. 3. Exon topology in the *A. thaliana* 3,4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II gene. The first and the last open bar correspond to the 5′ and 3′ untranslated regions, respectively.

The putative 3,4-dihydroxy-2-butanone 4-phosphate synthase domain of the *Arabidopsis* gene is preceded by a sequence of about 120 amino acids which is devoid of similarity to any sequence in the database. The first 25 amino acids comprise 13 serine residues (including a cluster of four consecutive serine residues). Thirty-four residues (28%) in the N-terminal 120 amino acid residues are serine or threonine.

Following the elucidation of the complete *ribA* cDNA sequence, the chromosomal *ribA* gene of *Arabidopsis* was amplified in two parts by PCR using chromosomal DNA as template. The amplificates were sequenced. The gene (EMBL database accession no. AJ000053) includes six introns with a total of 700 bp (Fig. 3). The 5′ and 3′ splice sites show homology to plant consensus sequences (Table 1). The introns contain a high proportion of adenine and uracil which is characteristic for dicot plants (Simpson & Filipowicz, 1996).

2.3. Complementation experiments

The open reading frame of the *A. thaliana ribA* gene was inserted into the expression vector pNCO113 yielding the plasmid pAE. This plasmid was electrotransformed into *ribB* and *ribA* mutant strains of *E. coli* which are deficient of 3,4-dihydroxy-2-butanone 4-phosphate synthase and GTP cyclohydrolase II, respectively, and which are therefore unable to grow on LB medium without added riboflavin (Katzenmeier, 1991). The plasmid pNCO113 transformed into the

Table 1
Splice sites of introns in the *ribA* gene of *A. thaliana*. Capital letters are part of open reading frames. Bold letters indicate similarity to the consensus sequence (5′, AGgtaagt, 3′ ttttttttttgcagGT)

	Length	5′-end	3′-end
Intron 1	267	**AGgtgag**g	ga**tgattctgtgcag**AC
Intron 2	81	**AGgttcgt**	gcgg**taatgttgcag**CT
Intron 3	87	**TGgtatgt**	ct**tccgcctccgtagG**A
Intron 4	90	**AGgtatgc**	**ttt**gaaaaa**tgacagGT**
Intron 5	92	**AGgttaga**	ct**gaaattgtgccagG**G
Intron 6	83	**AGgtatta**	**ttcttgaatctgcagAT**

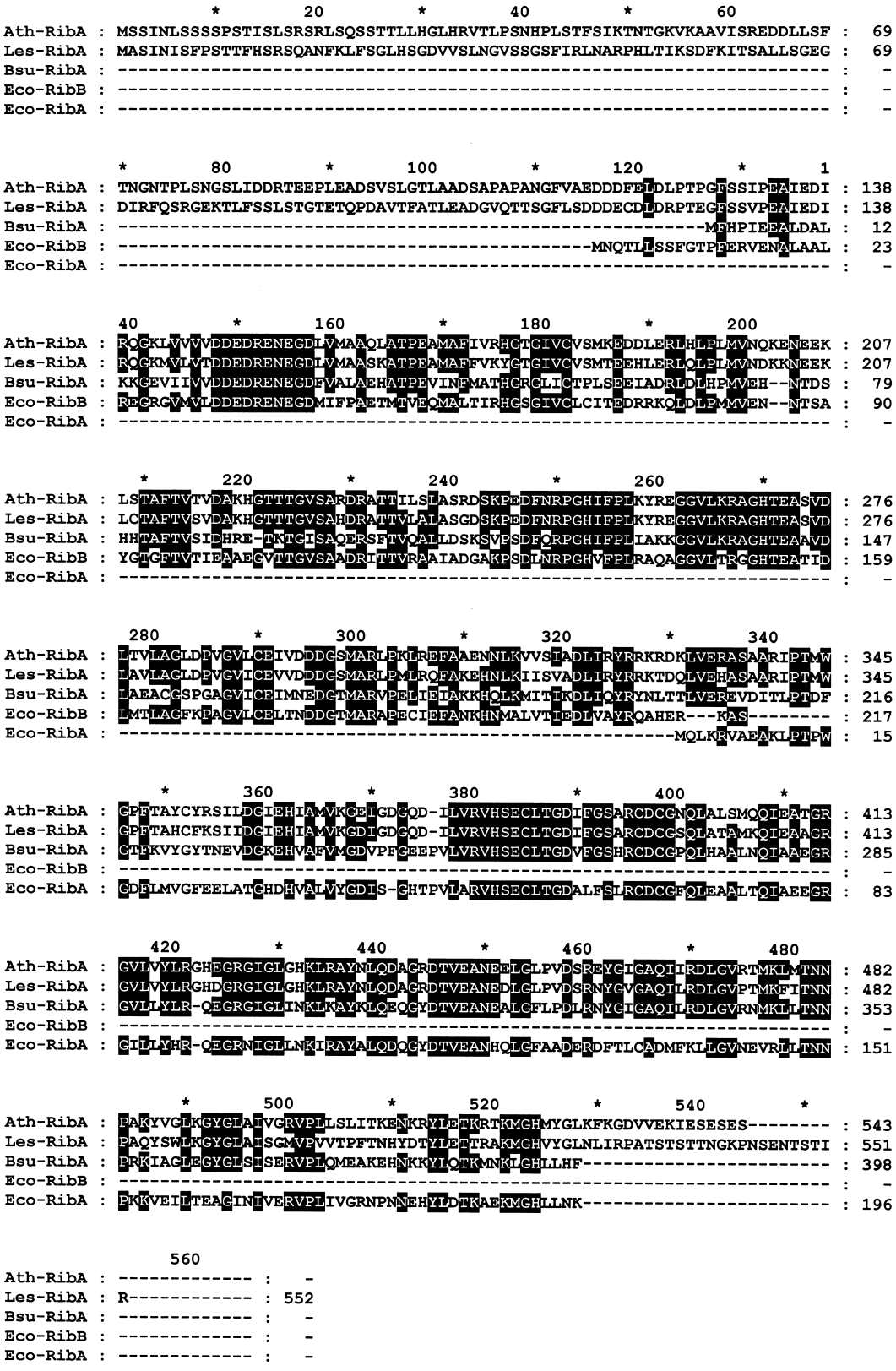

Fig. 4. Peptide sequence alignments. Ath-RibA and Les-RibA, bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase from *A. thaliana*, respectively, *L. esculentum*; Bsu-RibA, GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase from *B. subtilis*; Eco-RibA, GTP cyclohydrolase II from *E. coli*; Eco-RibB, 3,4-dihydroxy-2-butanone 4-phosphate synthase from *E. coli*.

mutants served as a negative control. The recombinant mutant strains carrying the plasmid with the *ribA* gene of *Arabidopsis* grow at normal rate in the absence of external riboflavin. It follows that the *ribA* gene of *Arabidopsis* directs the synthesis of a protein which can serve as GTP cyclohydrolase II and as 3,4-dihydroxy-2-butanone 4-phosphate synthase in *E. coli* cells. It follows that the *A. thaliana* enzyme is a bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase similar to that of *B. subtilis* (Fig. 4).

2.4. Cloning of the ribA gene of tomato

A gene with similarity to the *ribA* gene of *A. thaliana* was amplified from a tomato cDNA library by PCR using degenerate primers. The DNA segment was sequenced using a primer walk strategy and was shown to contain an open reading frame (EMBL database accession no. AJ002298) of almost the same size as the *A. thaliana* gene. The predicted protein sequence contains 552 amino acids and has a calculated mass of 59793 Da. Notably, the tomato protein also has a serine and threonine rich N-terminus of about 120 amino acids preceding the 3,4-dihydroxy-2-butanone 4-phosphate synthase domain. Apart from the high serine/threonine content, the N-terminus of the tomato gene has little similarity to the *A. thaliana* N-terminus. On the other hand, the sequence of the predicted catalytic 3,4-dihydroxy-2-butanone 4-phosphate synthase and GTP cyclohydrolase II domains from tomato and *Arabidopsis* enzyme shows 331 (82%) identical amino acids.

2.5. Expression of the ribA gene in E. coli

Whereas the plasmid pAE has been shown to complement the metabolic defects of *ribA* and *ribB* mutants of *E. coli*, the amount of the *Arabidopsis* protein expressed in the recombinant *E. coli* strain was very low. In an attempt to improve the expression level in the heterologous *E. coli* host, we constructed two plasmids specifying fusion proteins of *A. thaliana ribA* and maltose binding protein (MBP) of *E. coli*. For the construction of plasmid pMal-ribA, the *malE* gene was inserted at the 5′ end of the *Arabidopsis ribA* open reading frame. For the construction of plasmid pMal-ribAS, 127 codons of the *ribA* open reading frame were removed, and the *malE* gene was fused to the 5′ end of the shortened *ribA* gene. The fused genes were

Table 2
Purification of the maltose binding protein-ribA-fusion proteins. MBP-RibA, fusion protein with signal sequence; MBP-RibAS, fusion protein without signal sequence

Step	Enzyme activity[a] (U/mg)	
	MBP-RibA	MBP-RibAS
Cell extract	6	1
Sepharose Q	30	7
Amylose resin	165	53

[a] Only the GTP cyclohydrolase II activity was monitored.

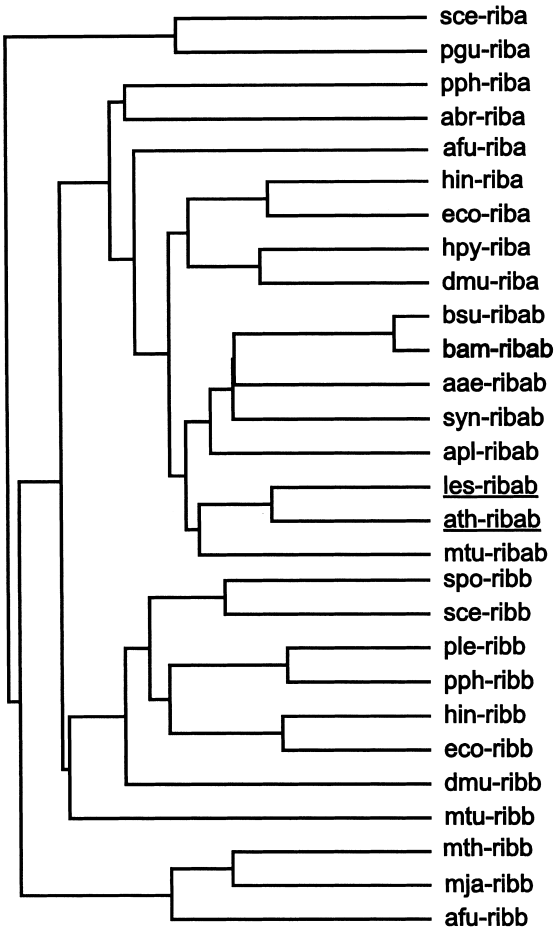

Fig. 5. Dendrogram of bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II of *Bacillus subtilis* (bsu-ribab), *Bacillus amyloliquefaciens* (bam-ribab), *Aquifex aeolicus* (aae-ribab), *Synechocystis sp.* (syn-ribab), *Actinobacillus pleuropneumoniae* (apl-ribab), *Lycopersicon esculentum* (les-ribab), *Arabidopsis thaliana* (ath-ribab) and *Mycobacterium tuberculosis* (mtu-ribab), of monofunctional GTP cyclohydrolase II of *Saccharomyces cerevisiae* (sce-riba), *Pichia guilliermondii* (pgu-riba), *Photobacterium phosphoreum* (pph-riba), *Azospirillum brasilense* (abr-riba), *Archaeoglobus fulgidus* (afu-riba), *Haemophilus influenzae* (hin-riba), *Escherichia coli* (eco-riba), *Helicobacter pylori* (hpy-riba) and *Dehalospirillum multivorans* (dmu-riba), and of monofunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase of *Schizosaccharomyces pombe* (spo-ribb), *S. cerevisiae* (sce-ribb), *Photobacterium leiognathi* (ple-ribb), *P. phosphoreum* (pph-ribb), *H. influenzae* (hin-ribb), *E. coli* (eco-ribb), *D. multivorans* (dmu-ribb), *M. tuberculosis* (mtu-ribb), *Methanobacterium thermoautotrophicum* (mth-ribb), *Methanococcus jannaschii* (mja-ribb) and *A. fulgidus* (afu-ribb). Dendrogram created with the program Pileup of the GCG Wisconsin Package. The sequences were retrieved from genbank.

under the control of a *tac* promoter and *lac* operator. Both plasmids directed the formation of fusion proteins which could be obtained in pure form by ion exchange chromatography followed by affinity chromatography on amylose resin (Table 2). Some properties of the purified proteins are summarized in Table 3.

The specific activities of the *A. thaliana* enzyme are substantially lower as compared to the homologous bacterial enzymes (Ritz, 1999; Bacher, Richter, Ritz, Eberhardt, Fischer & Krieger, 1997; Richter et al., 1997a). This may be in part due to the fusion with maltose binding protein. The *A. thaliana* fusion protein without the putative signal sequence (MBP-RibAS) is somewhat less active than the fusion protein containing the entire *Arabidopsis* open reading frame (MBP-RibA).

The GTP cyclohydrolase II activity of the *A. thaliana* RibA protein is only half as high as the 3,4-dihydroxy-2-butanone 4-phosphate synthase activity which might reflect the fact that only one molecule of 2,5-diamino-6-(β-D-ribosylamino)-4(3H)-pyrimidinone 5′-phosphate but two molecules of 3,4-dihydroxy-2-butanone 4-phosphate are needed for the synthesis of one molecule riboflavin. The *ribA* genes of *Arabidopsis* and tomato are 69.7% identical. Both plant genes resemble bacterial genes more closely than the yeast *rib1* and *rib3* genes (Fig. 5).

The N-terminal sections of the *Arabidopsis* and the tomato protein (about 120 amino acid residues) have no equivalents in the bacterial and yeast genes. Removal of the N-terminal segments does not change the enzyme activity significantly. The N-terminal sequence of the plant RibA proteins are likely to act as signal sequences for translocation into chloroplasts. Recently, it has been shown that lumazine synthase, catalyzing a later step in the biosynthesis of riboflavin is located in chloroplasts (Jordan et al., 1999).

3. Experimental

3.1. Materials

5-Amino-6-ribitylamino-2,4(1H, 3H)-pyrimidinedione was freshly prepared by catalytic hydrogenation of 5-nitro-6-ribitylamino-2,4(1H, 3H)-pyrimidinedione (Richter et al., 1997a). 6,7-Dimethyl-8-ribityllumazine was prepared as described by Bacher (1986). Oligonucleotides were purchased from MWG (Munich, Germany) and are summarized in Table 4.

3.2. Bacterial strains, media and plasmids

E. coli strain XL1 (Stratagene) was used for cloning and expression experiments. *E. coli* strains rib5 (*ribB*$^-$) and rib7 (*ribA*$^-$) (Katzenmeier, 1991) were used in complementation experiments. All *E. coli* strains were cultured in LB medium at 37°C. Ampicillin was added at a concentration of 150 mg/l and riboflavin was added at a concentration of 400 mg/l (dissolved at 65°C) when required.

Plasmid pGEM-T (Promega) was used for the subcloning of genomic *A. thaliana* DNA. Plasmid pNCO113 (Richter et al., 1997b) was used for gene expression in *E. coli*. Plasmid pMal-c2 (New England Biolabs) was used for the construction of the MBP-ribA-fusion protein. EST-clone 41G4T7 (Genbank account No. T04449) was obtained from the Arabidopsis Biological Resource Center, Ohio State University, USA.

3.3. Plasmid isolation and DNA sequencing

Plasmid was isolated from 5 ml of a fresh overnight culture using the mini plasmid isolation kit from Qiagen. Sequencing was performed by the automated dideoxynucleotide method (Sanger, Nicklen & Coulson, 1977).

3.4. Northern blotting

RNA was isolated from 3.6 g of two-weeks-old *A. thaliana* var. Columbia plants and was purified by centrifugation in a CsCl gradient (Logemann, Schell & Willmitzer, 1987). mRNA was isolated from this crude RNA fraction with the Oligotex mRNA-Isolation Kit. mRNA (4 μg) was subjected to electrophoresis on an 1% agarose gel containing 2.2 M formaldehyde and was transferred to a Nytrans nylon membrane.

A DNA segment comprising bp 39-1484 from

Table 3
Properties of maltose binding protein-ribA-fusion proteins. MBP-RibA, fusion protein with signal sequence; MBP-RibAS, fusion protein without signal sequence

Protein	MBP-RibA	MBP-RibAS
Predicted mass (kDa)	101.7	88.5
Apparent mass (SDS-PAGE) (kDa)	100	90
GTP cyclohydrolase II activity (nmol/h mg)	165	53
3,4-Dihydroxy-2-butanone 4-phosphate activity (nmol/h mg)	400	117

41G4T7 was labeled by the random priming method with [α–^{32}P]dCTP. Hybridization was performed at 42°C in a mixture containing 50% formamide and 50% SSPE/Denhards solution/SDS for 12 h (Denhardt, 1966). The membranes were then washed twice with 2 × SSPE (300 mM NaCl, 20 mM NaH_2PO_4, 2.5 mM EDTA, pH 7.4) containing 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min. The radioactive bands were detected for 3.5 h using a PhotoImager (Storm 860, Molecular Dynamics).

3.5. 5′ RACE

A 5′ RACE experiment was performed with the 5′ RACE system from Gibco BRL. The cDNA was generated using the primer ATU8 complementary to bp 533 to 555 of EST sequence 41G4T7. The cDNA was then amplified using the anchor primer RAP and the nested primer ATU10 complementary to bp 170 to 195 of the EST sequence 41G4T7. The PCR product was purified by electrophoresis in a 2% agarose gel.

3.6. Construction of subclones

The 5′ and 3′ parts of the *Arabidopsis* gene were amplified by PCR using the specific primers ATA1 and ATU9 respectively ARABR and ATU2N and chromosomal DNA from *A. thaliana* var. Columbia as template. The two overlapping PCR products corresponding to bp 2–1119 and bp 862–2837 were purified using the PCR purification kit from Quiagen. Each amplificate was ligated into T-tailed pGEM-T plasmids. After electrotransformation into *E. coli* XL1 cells according to the Biorad pulse controller manual, the clones were grown overnight in LB-medium.

3.7. Construction of an expression clone

Using plasmid isolated from EST clone 41G4T7 as template, the cDNA segment extending from bp −75 to 1484 of EST clone 41G4T7 was amplified by PCR with the primers Reverse and ARABE. The amplificate was purified with a PCR purification kit (Qiagen) and was digested with *Bsg*I. The RACE product was also digested with *Bsg*I, which cuts in the overlapping area. Both fragments were purified as before and were ligated with T4-Ligase at 4°C for 12 h. The resulting DNA was amplified by PCR. At the 5′ end, a recognition site for the restriction enzyme *Eco*RI preceding a ribosomal binding site at an optimal distance to the start codon was introduced by PCR with modifying primers EARV and BSECORI. At the 3′ end, a recognition site for the restriction enzyme *Sal*I was introduced after the stop codon by PCR with the primer EARH. The PCR product of 1697 bp was digested with the enzymes *Eco*RI and *Sal*I and was purified with the PCR purification kit (Qiagen). The expression vector pNCO113 was digested with the same restriction enzymes, purified and ligated with the insert, thus yielding plasmid pAE. The plasmid was transformed into *E. coli* XL1 cells by electroporation.

3.8. Amplification of the tomato ribA gene

A tomato cDNA library (18 ng) (Schmid, Schaller, Leibinger, Boll & Amrhein, 1992) was used as template for PCR with the degenerate primers PIV1 and PIH1.

Table 4
Oligonucleotides used for the construction of the ribA expression plasmids from *A. thaliana* and *L. esculentum*

Oligonucleotides	Primer sequence
ARABE	5′-TCAAGTTTCTCAGACAGATCAAATG-3′
ARABR	5′-CTGCTCCTGCACCAGCCAATGG-3′
ATA1	5′-GCAAGTGTCTTATCTCCTCCAC-3′
ATU2N	5′-CAAGAAGTAACAAATGATGAACATTTCG-3′
ATU8	5′-TCAACAGATGCTTCAGTGTGTCC-3′
ATU9	5′-GAGCAGCCATCACCAAATCC-3′
ATU10	5′-CCTTCATTTTCCCTATCTTCATCATC-3′
BSECORI	5′-CAATTTGAATTCATTAAAGAGGAGAAATTAACTATG-3′
EARH	5′-ACGCGTCGACGGTTCGTCCTGGTTTTTAAGC-3′
EARV	5′-GAGGAGAAATTAACTATGTCTTCCATCAATTTATCCTC-3′
MBMC	5′-GTTCAGAATTCATGTCTTCCATCAATTTATCCTC-3′
MBMCN	5′-GTTCAGAATTCTCTTCTATCCCCGAGGC-3′
PIH1	5′-TCRTTNGCTTCAACNGTRTC-3′
PIV1	5′-GAYGAAGAYMGNGAAAAYGAAGG-3′
RAP	5′-CUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′
Reverse	5′-GAAACAGCTATGACCATGATTACG-3′
TR1	5′-TGGTTCAGCCAGATGTGAC-3′
TU1	5′-CCTCTCCAAGTGTTCTTCTG-3′
Universe	5′-TTGTAAAACGACGGCCAGTG-3′

The amplificate was purified with a PCR purification kit (Qiagen) and sequenced. The unknown 5′ end of the gene was amplified by PCR with the primers TU1 and Reverse. The unknown 3′ end was amplified by PCR with the primers TR1 and Universe.

3.9. Expression of maltose binding protein-ribA-fusion proteins

The *ribA* gene of *A. thaliana* was amplified by PCR using plasmid pAE as template. A recognition site for the restriction enzyme *Eco*RI preceding the start codon was introduced at the 5′ end with the modifying primer MBMC and the exact primer EARH. The PCR product and the plasmid pMal-c2 were digested with the restriction enzymes *Sal*I and *Eco*RI, ligated together and electrotransformed into *E. coli* XL1 cells. The *malE-ribA* fusion clone without transit peptide sequence was constructed in the same way but the recognition site for the restriction enzyme *Eco*RI was introduced with primer MBMCN and EARH preceding phenylalanine at position 128.

3.10. Purification of fusion proteins

Recombinant *E. coli* strains were grown in 0.5 l LB-media containing 150 mg/l ampicillin at 37°C. At an optical density (600 nm) of 0.8, isopropylthiogalactoside was added to a final concentration of 1 mM. The cultures were incubated for another 5 h. The cells were harvested by centrifugation and were washed with 0.9% NaCl solution. Bacterial cell mass (3 g) was suspended in 10 ml 50 mM Tris hydrochloride pH 8.0 containing 6 mM phenylmethylsulfonyl fluoride and 0.1 mg/ml of lysozyme. The mixture was incubated for 1 h at 37°C with vigorous shaking and cooled on ice. Cells were disrupted by sonication. The suspension was centrifuged, and the supernatant was applied to a column of Sepharose Q (30 ml) which had been equilibrated with 50 mM Tris hydrochloride, pH 8.0. The column was developed with a gradient of 0–1 M NaCl containing 50 mM Tris hydrochloride pH 8.0. Fractions containing fusion-protein were combined, the pH was adjusted to 7.4. The NaCl concentration was adjusted to 200 mM, and the solution was applied to a column of amylose resin (2 ml, New England Biolabs), previously equilibrated with 20 mM Tris hydrochloride pH 7.4 containing 200 mM NaCl and 1 mM EDTA (buffer A). The column was washed with 12 volumes of buffer A. The fusion protein was eluted with 5 volumes of buffer A containing 10 mM maltose.

3.11. Assays of GTP cyclohydrolase II

Assay mixtures contained 100 mM Tris-hydrochloride pH 8.5, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM GTP, and protein in a total volume of 80 μl. They were incubated at 37°C for 30 min. A solution (20 μl) of 5% diacetyl in 250 mM EDTA was added. The mixture was incubated at 90°C for 1 h. 6,7-Dimethylpterin formed by reaction of the enzyme product with diacetyl was subsequently measured by reversed-phase HPLC using a column of Nucleosil RP18 (4 × 250 mm). The eluent contained 100 mM ammonium formate and 25% methanol. The effluent was monitored fluorometrically (excitation, 365 nm; emission, 435 nm). One unit of enzyme activity catalyzes the formation of 1 nmol of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate per h at 37°C.

3.12. Assay of 3,4-dihydroxy-2-butanone 4-phosphate synthase

Reaction mixtures contained 300 mM potassium phosphate, pH 7.5, 20 mM $MgCl_2$, 10 mM ribose 5-phosphate, and 0.1 U of pentose phosphate isomerase in a total volume of 30 μl. They were incubated at 37°C for 15 min, and 20 μl of the enzyme sample was added. The mixture was incubated for 30 min. A solution (50 μl) containing 200 mM potassium phosphate, pH 7.5, 40 mM dithiothreitol, 50 mM EDTA, 4 mM 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione, and 10 U of 6,7-dimethyl-8-ribityllumazine synthase was added. The mixture was incubated for 1 h. Trichloroacetic acid (50%, 100 μl) was added, and the mixture was centrifuged. 6,7-Dimethyl-8-ribityl-lumazine was determined by reversed-phase HPLC using a column of Nucleosil RP18 (4 × 250 mm). The eluent contained 30 mM formic acid and 10% methanol. The effluent was monitored fluorometrically (excitation, 408 nm; emission, 487 nm). One unit of enzyme activity catalyzes the formation of 1 nmol of 3,4-dihydroxy-2-butanone 4-phosphate per h at 37°C.

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