Physiologia Plantarum 126: 304–318. 2006 Copyright © Physiologia Plantarum 2006, ISSN 0031-9317

REVIEW

Biosynthesis of vitamin B_2 in plants

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Received 30 June 2005; revised 9 September 2005

doi: 10.1111/j.1399-3054.2005.00607.x

The biosynthesis of one riboflavin (vitamin B_2) molecule requires one molecule of GTP and two molecules of ribulose 5-phosphate. The imidazole ring of GTP is hydrolytically opened, yielding a 2,5-diaminopyrimidine that is converted to 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione by a sequence of deamination, side chain reduction and dephosphorylation. Condensation of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione with 3,4-dihydroxy-2-butanone 4-phosphate obtained from ribulose 5-phosphate yields 6,7-dimethyl-8-ribityllumazine. Dismutation of the lumazine derivative yields riboflavin and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione, which is recycled in the biosynthetic pathway. Characteristic architectural features of most enzymes involved in the plant riboflavin pathway resemble those of eubacteria, whereas the similarities between plants and yeasts are less pronounced. Moreover, riboflavin biosynthesis in plants proceeds by the same reaction steps as in eubacteria, whereas fungi use a somewhat different pathway.

Introduction

Flavocoenzymes are characterized by an extraordinary chemical versatility. They can catalyse not only redox processes involving one- and two-electron transitions but also a variety of non-redox reactions such as photorepair of thymidine dimers in photodamaged DNA (Imada et al., 2003; Sancar, 1994). More recently, they have been shown to act as chromophores in blue light photoreceptors of plants and fungi (Briggs et al., 2001; Christie et al., 1999; Lin et al., 1995). Moreover, they are involved in numerous other physiological processes involving bioluminescence and circadian time keeping (Lee, 1993; Meighen, 1993; Sancar, 2000, 2004).

Riboflavin (vitamin B_2; Fig. 1, **10**) is the universal precursor of the flavocoenzymes riboflavin phosphate (FMN, **11**) and flavin adenine dinucleotide (FAD, **12**). It is biosynthesised by plants and many microorganisms but must be obtained from dietary sources and/or the microbial gut flora by animals.

The investigation of the biosynthesis of the vitamin started around 1950. Ever since, practical aspects concerning the production of the vitamin for human and animal nutrition were a driving force for this investigation. Meanwhile, the chemical synthesis of the vitamin has been replaced by fermentation processes using bacteria or yeasts which yield more than 3000 metric tons per year.

Research on riboflavin biosynthesis has been predominantly conducted with microorganisms. More specifically, the early work was mainly focused on certain groups of fungi, notably Ascomycetes including *Eremothecium ashbyii* and *Ashbya gossypii* and yeasts

Abbreviations – GTP, Guanosine 5′-triphosphate; GMP, Guanosine 5′-monophosphate; FMN, flavin mononucleotide; FAD, flavin-adenine dinucleotide; GCYHI, GTP cyclohydrolase II; DHBPS, 3,4-dihydroxy-2-butanone 4-phosphate synthase; NMR, nuclear magnetic resonance; DEAM, 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate deaminase; RED, 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione 5′-phosphate reductase.

including several different *Candida* spp. The reason for this preference was the natural occurrence of moderately flavinogenic representatives of these groups. More recently, certain eubacteria including *Escherichia coli* and *Bacillus subtilis* that are not naturally flavinogenic, along with the non-flavinogenic yeast, *Saccharomyces cerevisiae*, became preferred objects of riboflavin biosynthesis research. By comparison, the study of riboflavin biosynthesis in plants was essentially a neglected area, despite the fact that plants play a dominant role in the supply of animals with riboflavin. Apart from some work on the terminal enzymes of the riboflavin pathway, lumazine synthase and riboflavin synthase, the study of riboflavin biosynthesis in plants has only been initiated on a broader scale during the last decade.

For a long period, many biochemists were inclined to believe that the study of plants could not add substantially to the understanding of metabolic pathways that had been investigated in significant detail in microorganisms and/or mammals. A major surprise from the recent work on the biosynthesis of riboflavin in plants was the finding that the plant pathway has much closer similarity with the eubacterial pathway as compared with archaea and fungi.

The work on riboflavin biosynthesis in microorganisms has been covered extensively in recent reviews (Bacher et al., 2000, 2001; Fischer and Bacher, 2005; Fischer et al., 2005b).

Fig. 1 shows a summary of the current state of information on riboflavin biosynthesis. The pathway starts off by the hydrolytic release of formate and inorganic pyrophosphate from GTP (**1**) which is catalysed by GTP cyclohydrolase II (Fig. 1, step **I**). The product, 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate (**2**), is converted into 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione 5′-phosphate (**5**) via 2,5-diamino-6- ribitylamino-4(3*H*)-

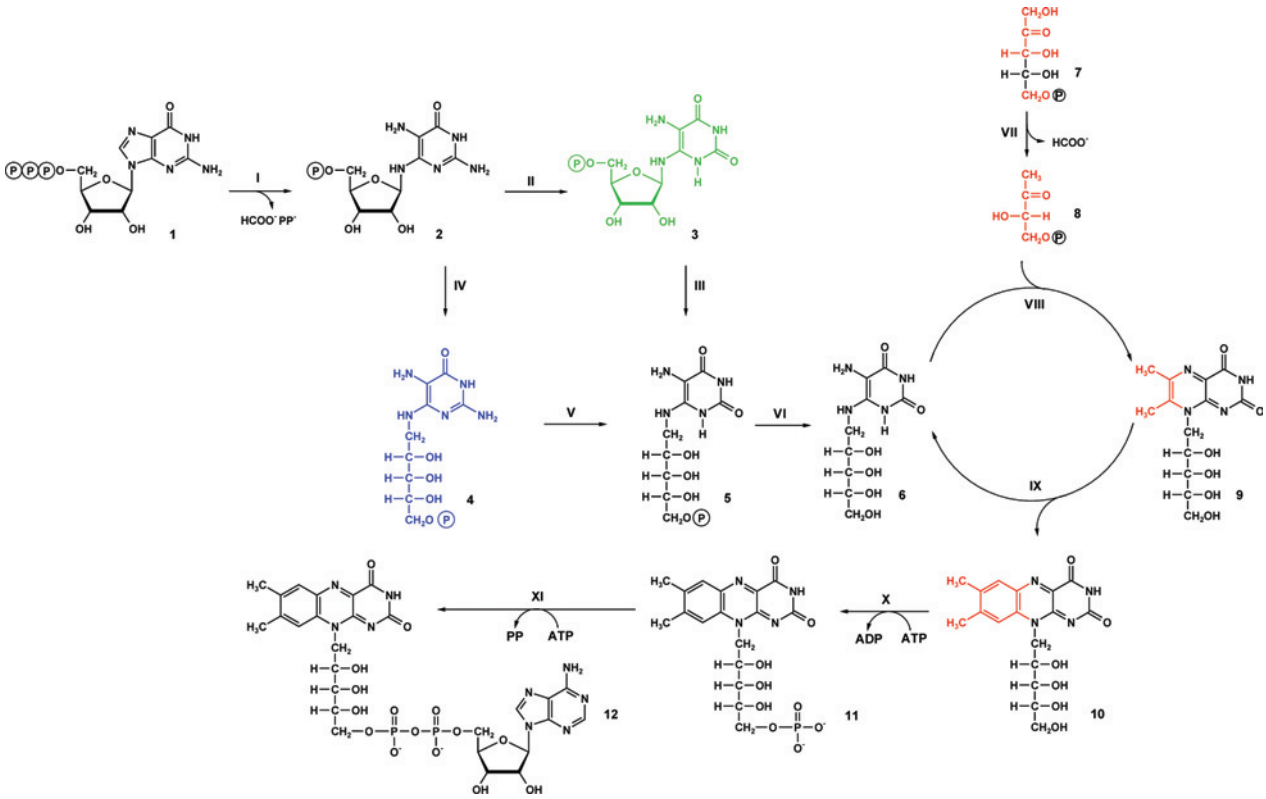

Fig. 1. Biosynthesis of riboflavin and flavocoenzymes. Step **I**, GTP cyclohydrolase II; step **II**, 2,5-diamino-6-ribosylamino-4 (3*H*)-pyrimidinone 5′-phosphate deaminase; step **III**, 5-amino-6-ribosylamino-2,4 (1*H*,3*H*)-pyrimidinedione 5′-phosphate reductase; step **IV**, 2,5-diamino-6-ribosylamino-4 (3*H*)-pyrimidinone 5′-phosphate reductase; step **V**, 2,5-diamino-6-ribitylamino-4 (3*H*)-pyrimidinedione 5′-phosphate deaminase; step **VI**, hypothetical phosphatase; step **VII**, 3,4-dihydroxy-2-butanone 4-phosphate synthase; step **VIII**, 6,7-dimethyl-8-ribityllumazine synthase; step **IX**, riboflavin synthase; step **X**, riboflavin kinase; step **XI**, FAD synthetase; **1**, GTP; **2**, 2,5-diamino-6-ribosylamino-4 (3*H*)-pyrimidinone 5′-phosphate; **3**, 5-amino-6-ribosylamino-2,4 (1*H*,3*H*)-pyrimidinedione 5′-phosphate; **4**, 2,5-diamino-6-ribitylamino-4 (3*H*)-pyrimidinedione 5′-phosphate; **5**, 5-amino-6-ribitylamino-2,4 (1*H*,3*H*)-pyrimidinedione 5′-phosphate; **6**, 5-amino-6-ribitylamino-2,4 (1*H*,3*H*)-pyrimidinedione; **7**, ribulose 5-phosphate; **8**, 3,4-dihydroxy-2-butanone 4-phosphate; **9**, 6,7-dimethyl-8-ribityllumazine; 10, riboflavin; 11, FMN; 12, FAD. Green, eubacterial/plant pathway; blue, fungal/archaeal pathway; red, fate of the four-carbon precursor **8** derived from ribulose 5-phosphate.

pyrimidinedione 5′-phosphate (**4**) in archaea and in fungi or via 5-amino-6-ribosyl amino-2,4(1*H*,3*H*)-pyrimidinedione 5′-phosphate (**3**) in eubacteria and plants (Fig. 1, steps **II** and **III** or **IV** and **V**).

5-Amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione 5′-phosphate (**5**) is dephosphorylated by a hitherto unknown process (Fig. 1, step **VI**). The resulting pyrimidine derivative **6** yields 6,7-dimethyl-8-ribityllumazine (**9**) by condensation with 3,4-dihydroxy-2-butanone 4-phosphate (**8**) which is obtained from ribulose 5-phosphate (**7**) by a skeletal rearrangement. The final step of the biosynthetic pathway involves an unusual dismutation of the pteridine derivative **9** yielding riboflavin (**10**) and the pyrimidine **6**. The fate of the sugar precursor in the products, 6,7-dimethyl-8-ribityllumazine (**9**) and riboflavin (**10**), is shown in red in Fig. 1.

Although the early intermediates of the riboflavin pathway are 5′-phosphoric acid esters, the product of the biosynthetic pathway is unphosphorylated riboflavin. Hence, phosphorylation of riboflavin by riboflavin kinase (Fig. 1, step **X**) is invariably required in prototrophic as well as in auxotrophic species to obtain FMN (**11**) and FAD (**12**).

The following sections describe the genes and enzymes of riboflavin biosynthesis in plants. Supplementary information on microbial enzymes will be introduced to better illustrate aspects that have not been studied with the plant proteins.

GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase

The publication of the complete *Arabidopsis thaliana* genome enabled the rapid identification of numerous open reading frames by comparison with known genes from eubacteria and fungi. This approach identified the *ribAB* gene of *A. thaliana* based on its similarity with homologous genes from *B. subtilis* and *E. coli* (Herz et al., 2000). More specifically, the similarity with the *A. thaliana ribAB* gene extends almost over the entire length of the orthologous *B. subtilis* gene. On the other hand, the *ribA* gene of *E. coli* was similar to the C-terminal part of the plant gene, whereas a major section of the N-terminal part is similar to the *ribB* gene of *E. coli*.

The *ribA* and *ribB* genes of *E. coli* specify GTP cyclohydrolase II (Fig. 1, step **I**; GCHYII) and 3,4-dihydroxy-2-butanone 4-phosphate synthase (Fig. 1, step **VII**, DHBPS); the *ribA* gene of *B. subtilis* specifies a bifunctional enzyme with both activities. Hence, there could be little doubt that the enzyme specified by the plant gene should catalyse both initial reactions of the convergent riboflavin pathway.

An N-terminal segment of the *A. thaliana* enzyme comprising about 120 amino acid residues shows no similarity at all with any bacterial enzyme. Thirty-four residues (28%) in the N-terminal 120 amino acid residues are serine or threonine. The N-terminal sections of the *Arabidopsis* and the *tomato* protein have no equivalents in the bacterial and yeast kingdoms; they are believed to act as signal sequences for translocation into chloroplasts (Herz et al., 2000).

A pseudomature sequence without the putative targeting sequence fused to the C-terminus of maltose binding protein of *E. coli* could be expressed in a recombinant *E. coli* strain. Studies with the purified recombinant protein confirmed the expected bifunctionality. The specific activities of the *A. thaliana* enzyme are substantially lower as compared with the homologous bacterial enzymes (Bacher et al., 1997; Richter et al., 1993, Ritz et al., 2001). This might be in part due to the fusion with maltose binding protein which was performed in an attempt to facilitate expression in the heterologous *E. coli* host.

The bifunctional plant protein has not been studied in significantly more detail up to now. On the other hand, orthologous GCYHII and DHBPS of microbial origin have been studied in considerable detail; these studies have been reviewed elsewhere (Fischer and Bacher, 2005).

The reaction mechanism of GCYHII has been studied predominantly with the *E. coli* enzyme (Foor and Brown, 1975, 1980). Presteady-state kinetic analysis suggests that GTP forms a covalent adduct with the protein under the loss of inorganic pyrophosphate, but the respective amino acid serving as a nucleophile for adduct formation has not yet been identified (Ritz et al., 2001). The hypothetical covalent adduct can be cleaved under the formation of GMP in a side reaction (Ritz et al., 2001). Alternatively, the imidazole ring of the covalently bound guanyl moiety can be opened by hydrolytic cleavage of the bond between C-8 and N-7 yielding the formamide **16** (Fig. 2).

Hydrolytic cleavage of the formamide bond of **16** yields formate (Fig. 2, **17** and **18**). Both hydrolysis steps require a zinc ion that is believed to be complexed by three cysteine residues (Kaiser et al., 2002). The reaction is terminated by the cleavage of the phosphodiester bond. The three-dimensional structure of GCYHII remains to be determined; the mechanistic information is based on presteady-state kinetic analysis in conjunction with site-directed mutagenesis (Kaiser et al., 2002; Ritz et al., 2001; Schramek et al., 2001).

The mechanism of the second reaction catalysed by the bifunctional GCYHII/DHBPS is also characterised by extraordinary complexity (Fig. 3). Again, the

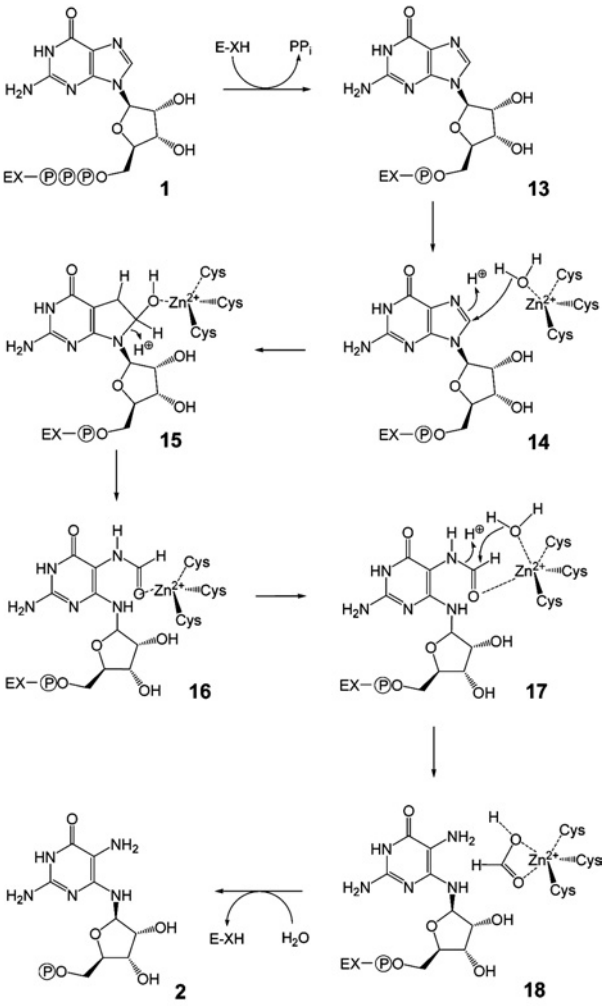

Fig. 2. Hypothetical mechanism for release of formate by GTP cyclohydrolase II (Fig. 1, step **I**) (Kaiser et al., 2002).

available information is based on studies with microbial orthologues. Briefly, the formation of 3,4-dihydroxy-2-butanone 4-phosphate (**8**) is believed to be initiated by the release of water from ribulose 5-phosphate (**7**). The resulting diketone has been proposed to undergo a Lobry de Bryn isomerisation yielding the branched aldose **23**. The subsequent release of formate is followed by keto-enol tautomerisation yielding 3,4-dihydroxy-2-butanone 4-phosphate. The hypothetical reaction mechanism suggests a crucial role for acid/base catalysis, and polar ligands are probably involved in the interaction of the protein with the essential divalent Mg^{2+} ion (Volk and Bacher, 1990).

The structure of the *E. coli* enzyme has been studied in considerable detail by X-ray diffraction and nuclear magnetic resonance (NMR) spectroscopy (Kelly et al., 2001; Liao et al., 2001a). X-ray structures were also reported for the enzymes from *Magnaporthe grisea*, *Methanococcus jannaschii* and *Candida albicans* (Echt et al., 2004; Liao et al., 2002; Steinbacher et al., 2003, 2004) (Fig. 4).

The active site could be localized by crystallographic analysis of the enzymes from *M. jannaschii* and *C. albicans* in complex with ribulose phosphate (Echt et al., 2004; Steinbacher et al., 2003, 2004). A highly conserved loop comprising several acidic amino acid residues is essential for catalysis as shown by studies with a variety of mutant proteins (Fischer et al., 2002).

Deaminase and reductase

The conversion of 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinone 5′-phosphate (**2**) into 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione 5′-phosphate (**5**) had

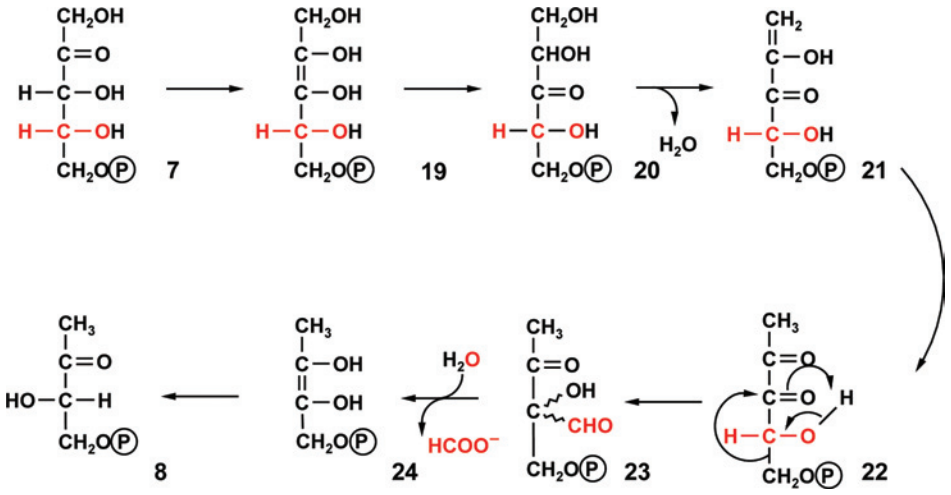

Fig. 3. Hypothetical reaction mechanism of 3,4-dihydroxy-2-butanone 4-phosphate synthase (Fig. 1, step **VII**) (Fischer et al., 2002; Steinbacher et al., 2003; Volk and Bacher, 1990).

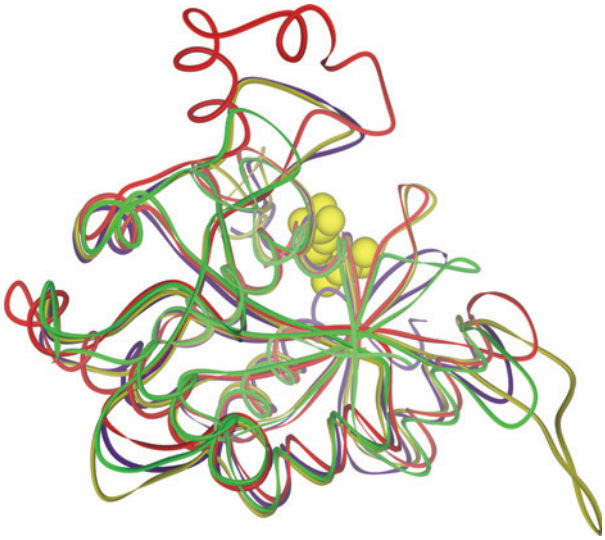

Fig. 4. Overlay of single subunits of 3,4-dihydroxy-2-butanone 4-phosphate synthases of *Candida albicans* (green), *Escherichia coli* (blue), *Methanococcus jannaschii* (red) and *Magnaporthe grisea* (olive green). Only the substrate ribulose 5-phosphate (**7**) of the *C. albicans* complex is shown (yellow) (Echt et al., 2004; Liao et al., 2001, 2002; Steinbacher et al., 2003; 2004).

been shown earlier to proceed via different intermediates in fungi and bacteria (Bacher and Lingens, 1970; Burrows and Brown, 1978; Nielsen and Bacher, 1981). In yeasts, the reaction sequence is initiated by the reductive conversion of the ribosyl side chain of **2** into the ribityl side chain of 2,5-diamino-6-ribitylamino-4(3*H*)-pyrimidinedione 5′-phosphate (**4**); subsequent deamination of the pyrimidine moiety yields **5**. The reactions are catalysed by independent proteins. In bacteria, on the other hand, the reaction sequence begins with the deamination of **2** yielding 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione 5′-phosphate (**3**). That reaction and the subsequent side chain reduction of **3** are catalysed by bifunctional fusion proteins consisting of 2,5-diamino-6-ribosylamino-4 (3*H*)-pyrimidinone 5′-phosphate deaminase (Fig. 1, step **II**, DEAM) and 5-amino-6-ribosylamino-2,4 (1*H*,3*H*)-pyrimidinedione 5′-phosphate reductase (Fig. 1, step **III**, RED) domains in the majority of completely sequenced eubacteria (Richter et al., 1997). In bacteria as well as in yeasts, the intermediate **5** must become dephosphorylated by a hitherto unknown process.

The recent discovery, in *A. thaliana*, of an enzyme with close similarity to the bacterial DEAM-domain of a bifunctional DEAM/RED protein was a considerable surprise. Briefly, the open reading frame At4g20960, designated *rib2*, was expressed in a recombinant bacterial host and was shown to yield a protein of 39.7 kDa catalysing the deamination of **2**. The reaction was studied in some detail by NMR spectroscopy using ^{13}C-labelled substrate. The deaminase requires zinc ions for activity (Fischer et al., 2004b). These data clearly showed that plants which belong to eukaryotes use the same pathway as eubacteria (i.e. via **3**, Fig. 1) for the transformation of **2** into **5**. On the other hand, fungi use a different pathway (via **4**, Fig. 1). However, in contrast to the bifunctional eubacterial enzymes, the plant enzyme is a monofunctional deaminase. The plant enzymes required for the side chain reduction of the deaminase product **3** (RED) remains to be discovered.

All known *rib2* genes from plants predict N-terminal peptide segments that fulfil the criteria for targeting sequences. The sequences of the catalytic domains of all plant enzymes are closely similar, whereas the putative targeting sequences are devoid of significant sequence similarity (Fischer et al., 2004b).

Although the structure of the plant DEAM has not been determined, the X-ray structure of the paralogous yeast cytosine deaminase has been solved in the presence of an inhibitor at 1.14 Å resolution (Ireton et al., 2003). In this enzyme, each active site contains a single catalytic zinc ion which is coordinated by one histidine, two cysteines and a single bound water molecule. An identical coordination site for the zinc ion is also found and strictly conserved over 54 bacterial and nine plant DEAMs (Fig. 5). Functionally crucial residues as derived from the cytosine deaminase structure are also present in the plant DEAM and appear to have been conserved in all plant orthologues whose sequences have been obtained. Thus, the mechanism of deamination appears similar in this enzyme superfamily.

6,7-Dimethyl-8-ribityllumazine synthase

The *ribH* gene specifying the penultimate enzyme in the riboflavin synthase pathway, 6,7-dimethyl-8-ribityllumazine synthase (Fig. 1, step **VIII**, lumazine synthase), has been cloned from spinach by a marker rescue strategy using a mutant of *E. coli* (Jordan et al., 1999). Subsequently, the genes for several plant orthologues have been reported. The three-dimensional structure of the spinach enzyme has been determined by X-ray crystallography (Persson et al., 1999) and will be described in more detail below.

Lumazine synthases from bacteria, fungi and yeast are all similar with regard to their sequences and enzymatic properties (Jordan et al., 1999).

Detailed mechanistic studies performed with the *B. subtilis* enzyme suggested the reaction mechanism summarised in Fig. 6. Briefly, the reaction starts with the formation of a Schiff's base by reaction of the

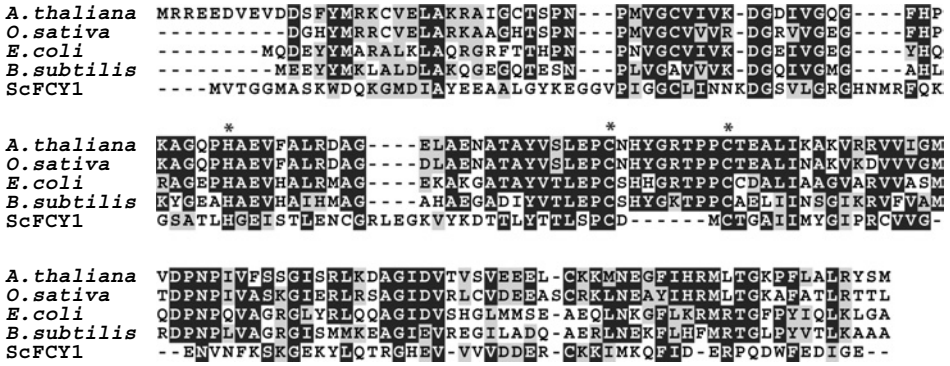

Fig. 5. Sequence alignment of 2,5-diamino-6-ribosylamino-4 (3*H*)-pyrimidinone 5′-phosphate deaminase domains from eubacteria and plants with cytosine deaminase from *Saccharomyces cerevisiae*. *Arabidopsis thaliana* (At4g20960); *Bacillus subtilis* ribG (P17618); *Escherichia coli* ribD (Q8FKC3); *Oryza sativa* (AK070281); and *ScFCY1* (cytosine deaminase of *S. cerevisiae*) (Q12178). The highly conserved zinc coordination site of yeast cytosine deaminase is indicated by asterisk (Fischer et al., 2004b).

position 5 amino group of **6** with the carbonyl group of **8** (Keller et al., 1988; Kis et al., 1995; Nielsen et al., 1986). The elimination of phosphate prepares the stage for the formation of the lumazine chromophore by ring closure. Notably, several mechanistic variations on this general theme are possible and have not been ruled out explicitly.

Remarkably, the reaction mechanism in Fig. 6 suggests that proton transfer reactions should play an important role. However, a detailed mutation study performed with the *B. subtilis* enzyme failed to identify any specific amino acid residue that is essential for the reaction (Fischer et al., 2003a). Hence, it was concluded that the enzyme operates predominantly by controlling the reaction entropy. It is relevant in that context that the activation barrier for the condensation of **6** with **8** is quite low. In fact, the reaction proceeds at appreciable rates at room temperature in neutral aqueous solution without enzyme, and the catalytic acceleration by lumazine synthase is quite modest (Fischer et al., 2003a; Haase et al., 2003).

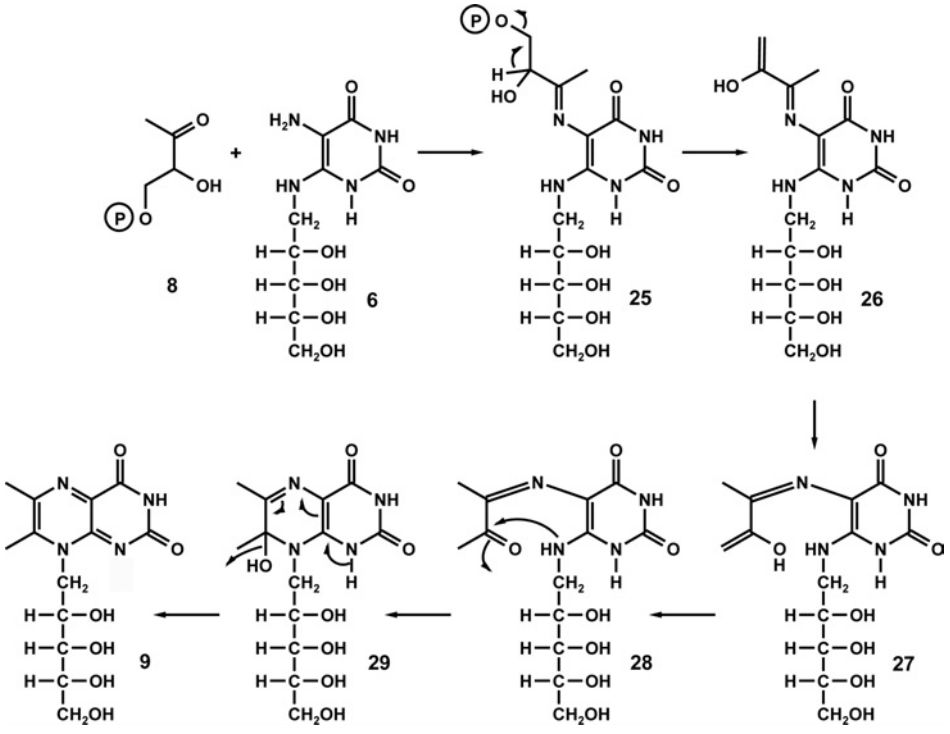

Fig. 6. Hypothetical reaction mechanism of lumazine synthase (Fig. 1, step **VIII**) (Kis et al., 1995).

Structures of lumazine synthases from plant, eubacterial, fungal and archaeal origin have been studied in considerable detail by X-ray crystallography and electron microscopy (Bacher et al., 1986, 1994; Braden et al., 2000; Gerhardt et al., 2002a; Koch et al., 2004; Ladenstein et al., 1988, 1994; Meining et al., 2000; Morgunova et al., 2005; Mörtl et al., 1996; Persson et al., 1999; Ritsert et al., 1995; Zhang et al., 2001, 2003; Zylberman et al., 2004). The proteins form either c_5-symmetric homopentamers (yeasts and certain eubacteria) or capsids of 60 identical subunits characterized by icosahedral 532 symmetry, which are best described as dodecamers of pentamers (plants, archaea and many eubacteria). The subunit folds are all closely similar (Fig. 7).

The topologically equivalent active sites (five in case of the pentameric enzymes and 60 in case of the icosahedral enzymes, Fig. 7) are all located at the interfaces between adjacent subunits in the pentamer motif. The structural complexity of some of these proteins is in surprising contrast with the absence of any amino acid residues that are individually of major importance for the enzyme-catalysed reaction (Fischer et al., 2003a).

In *Bacillaceae*, lumazine synthase and riboflavin synthase form a complex comprising an icosahedral capsid of 60 lumazine synthase subunits and a core of three riboflavin synthase subunits; historically, these unusual enzyme complexes were designated heavy riboflavin synthase (Bacher and Mailänder, 1978; Bacher et al., 1980). The topological features of the bifunctional enzyme complex are conducive to enhanced overall reaction rates via substrate channelling under certain reaction conditions (Kis and Bacher, 1995). At present, it is unknown whether this unusual quaternary structure occurs in plants.

Riboflavin synthase

Whereas the early enzymes of the riboflavin pathway were only obtained from plants during the last decade, riboflavin synthase (Fig. 1, step **IX**) had been partially purified from spinach in the 1960s (Mitsuda et al., 1970). More recently, riboflavin synthase from *A. thaliana* has been obtained by heterologous expression in *E. coli* cells. Sequence arguments show that the enzyme has the typical two-domain architecture of riboflavin synthases from bacteria and fungi (Fischer et al., 2005a).

The enzyme sediments at an apparent velocity of 3.9 S at 20°C like orthologous riboflavin synthases of various microorganisms. The purified *A. thaliana* riboflavin synthase was characterised by bright yellow colour that was caused by non-covalent binding of riboflavin with a K_D value of 1.1 μ*M* with one molecule of riboflavin bound per subunit. In contrast, most other riboflavin synthases studied so far were isolated without bound riboflavin, indicating a significantly lower affinity (Fischer et al., 2005a).

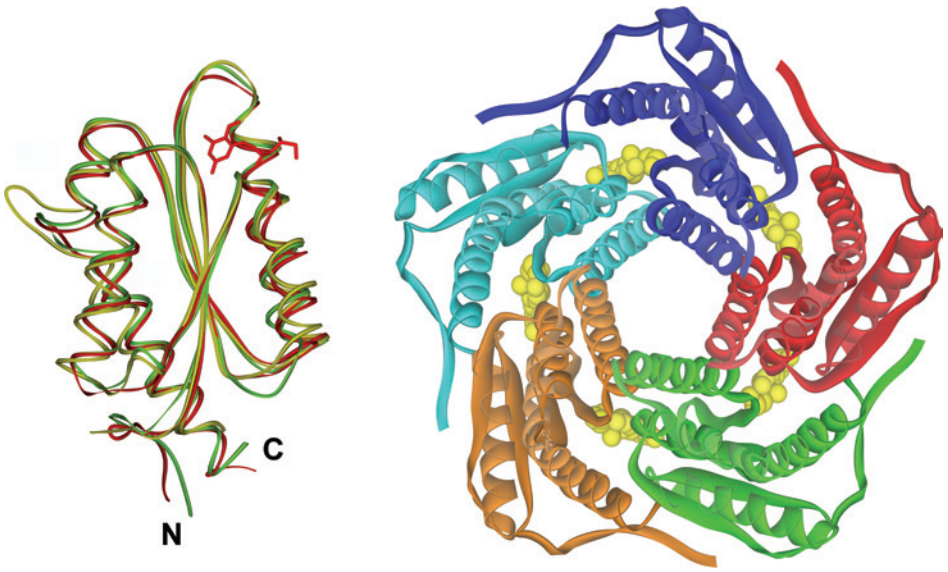

Fig. 7. Left: structural superposition of monomers of the pentameric lumazine synthase from *Schizosaccharomyces pombe* (yellow) and the icosahedral lumazine synthases from *Aquifex aeolicus* (green) and spinach (red). A bound substrate-analogue inhibitor 5-nitro-6-(D-ribitylamino)-2,4 (1*H*,3*H*)-pyrimidinedione, to mark the substrate binding site is shown (based on the spinach structure) (Gerhardt et al., 2002a; Persson et al., 1999; Zhang et al., 2003). Right: pentameric assembly of *spinach* lumazine synthase viewed along the five-fold non-crystallographic symmetry axis as seen from the inner capsid wall. The active sites are built up by two adjacent monomers. Bound ligand is shown in yellow (Persson et al., 1999).

The plant riboflavin synthases are similar to the eubacterial and fungal orthologues, but the latter have been studied in much closer detail (Fischer et al., 2003b; Gerhardt et al., 2002b). Hence, the following sections are primarily based on data obtained with riboflavin synthases of eubacterial and fungal origin.

The reaction catalysed by riboflavin synthase can be formally described as a dismutation involving the transfer of a four-carbon moiety between two identical substrate molecules (Paterson and Wood, 1969, 1972; Plaut, 1971). The second product of that dismutation, **6**, serves as the substrate for the penultimate step of riboflavin biosynthesis and is recycled by lumazine synthase (Fig. 1, step **VIII**). By their joint action, lumazine synthase and riboflavin synthase generate one equivalent of riboflavin from one equivalent of GTP (**1**) and two equivalents of ribulose 5-phosphate (**7**). Interestingly, the formation of riboflavin from the lumazine derivative **9** can proceed in aqueous solution under neutral or acidic conditions in the absence of any catalyst. The acidic protons of the position 7 methyl group are easily exchanged with solvent water (Beach and Plaut, 1970; Paterson and Wood, 1969; Plaut et al., 1970), and this exchange is accelerated by riboflavin synthase (Plaut et al., 1970).

Recent studies have shown that the initial reaction steps are conducive to the formation of a pentacyclic adduct of two substrate molecules (Illarionov et al., 2001a). Earlier studies had already shown that the two four-carbon fragments yielding the xylene ring of the vitamin are combined with an antiparallel orientation (Fig. 8).

The reaction steps conducive to the adduct formation are still a matter of considerable speculation, but it appears likely that the unusual acidity of the position 7 methyl group of the substrate **9** (with a pK of 8.5) is an important factor (Pfleiderer and Hutzenlaub, 1973). In contrast to the mechanistic intricacies of the formation of the pentacyclic adduct, its cleavage by a sequence of β-eliminations presents no particular mechanistic problems.

The riboflavin synthases of plants, eubacteria and fungi are all characterized by a high degree of intramolecular sequence similarity as shown in Fig. 9. A folding pattern of two domains with close topologic similarity was initially predicted on basis of sequence arguments and was subsequently confirmed by structural studies (Gerhardt et al., 2002b; Liao et al., 2001b; Schott et al., 1990). This two-domain architecture has important implications for the dismutation mechanism.

The structures of riboflavin synthase from *E. coli* and the yeast, *Schizosaccharomyces pombe*, have been determined by X-ray crystallography (Gerhardt et al.,

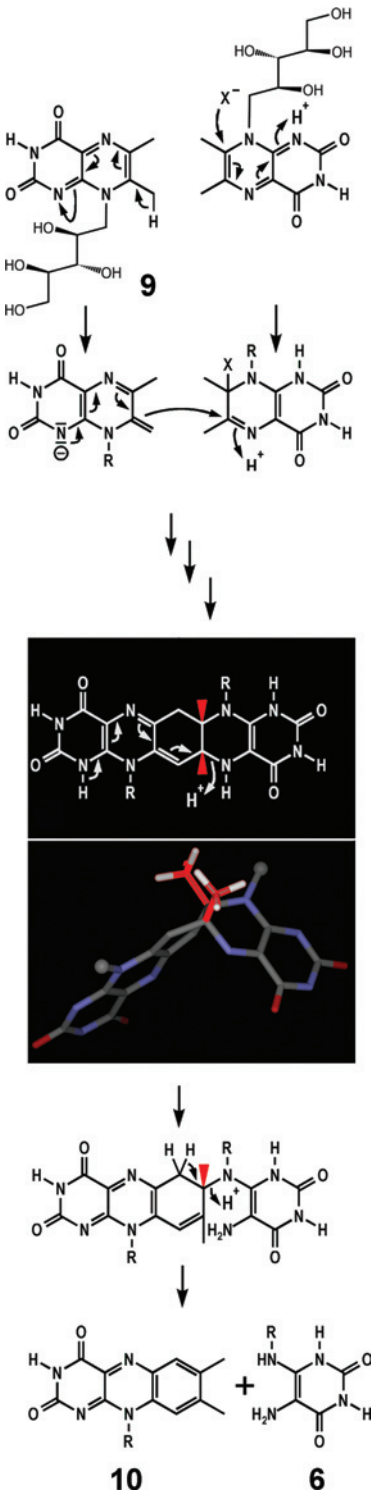

Fig. 8. Hypothetical reaction mechanism of riboflavin synthase: **6**, 5-amino-6-ribitylamino-2,4 (1*H*,3*H*)-pyrimidinedione; **9**, donor and acceptor lumazine molecules; **10**, riboflavin; **X**$^-$, proposed nucleophile, which neutralizes the carbonium centre at C-7 of **9** and enables carbanion attack at C-6 by the 7-exomethylene carbon of **9**; **R**, ribityl chain; black, pentacyclic intermediate (Illarionov et al., 2001a, 2005).

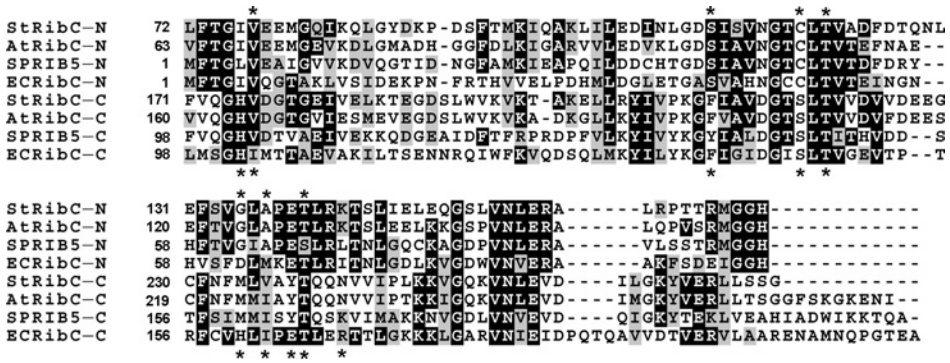

Fig. 9. Intramolecular sequence similarity of riboflavin synthase subdomains from *Arabidopsis thaliana* and *Solanum tuberosum* (without targeting sequence), *Escherichia coli* and *Schizosaccharomyces pombe*. N, N-terminal domain; C, C-terminal domain. Identical residues are shaded in black; similar residues are highlighted in grey. Residues which are believed to interact with the substrate (Gerhardt et al., 2002b) are marked by asterisk.

2002b; Liao et al., 2001b). An artificial dimer of the recombinant N-terminal domain of the *E. coli* enzyme has been studied by X-ray crystallography and by NMR spectroscopy (Meining et al., 2003; Truffault et al., 2001). These studies all confirm the close similarity between the folding patterns of the N-terminal and C-terminal domains. Each of the domains can bind one substrate molecule in a shallow cavity. The active site is formed at the interface of the N-terminal domain of one subunit (which serves as the four-carbon donor site) and the C-terminal domain of an adjacent subunit (serving as four-carbon acceptor site). At the interface between the respective N-terminal and C-terminal domains of two adjacent subunits in the homotrimer, the two respective substrate binding sites are in close proximity. Moreover, the two bound substrate molecules in that configuration are related by pseudo-c_2 symmetry (dictated by the pseudo-c_2 symmetry of the protein environment) that is required by the antiparallel regiospecificity of the enzyme-catalysed reaction (Gerhardt et al., 2002b; Liao et al., 2001b). The pseudo-c_2-symmetric domain pair revealed by the crystallographic analysis is well in line with the current understanding of the reaction mechanism via the pentacyclic intermediate described above (Fig. 8).

A detailed mutagenesis study failed to assign any specific amino acid residues at the active site to specific aspects of the catalytic mechanism (Fischer et al., 2003b; Illarionov et al., 2001b). Although almost any changes in the N-terminal pattern involving two hydrophobic amino acid residues (methionine and phenylalanine) are highly detrimental to the enzyme activity, these residues are only involved in substrate positioning but not in catalysis per se. A significant reduction of enzyme activity is caused by the replacement of a serine residue (S146 in the *E. coli* enzyme), but once again this cannot be correlated in a straightforward way with a specific catalytic function (Fischer et al., 2003b; Illarionov et al., 2001b). Other amino acid residues in the first shell around the active site can be exchanged without a significant penalty. This is in part due to the fact that backbone atoms play a dominant role for substrate binding, as opposed to side chain interactions. In summary, the data suggest that the control of entropic factors is a dominant aspect for riboflavin synthase catalysis, in close similarities with the situation described above for lumazine synthase.

Recently, a riboflavin synthase without apparent sequence similarity to the enzymes from eubacteria, fungi and plants has been cloned and characterized from *Methanobacterium thermoautotrophicum* and *M. jannaschii* (Eberhardt et al., 1997; Fischer et al., 2004a; Illarionov et al., 2005). Although the pseudo-c_2-symmetry of the respective N-terminal and C-terminal domains that together form the active site of classical riboflavin synthases (e.g. of *E. coli*) is by necessity conducive to the required quasi-c_2-topology of the substrate molecules, the archaeal type riboflavin synthases are devoid of any symmetry properties that would inherently enforce the required symmetry properties of the substrate topology. Interestingly, those riboflavin synthases are paralogues of lumazine synthase (Fischer et al., 2004a).

Riboflavin kinase

Flavin mononucleotide (FMN, riboflavin 5′-phosphate, **11**) and FAD (Fig. 1, **12**) serve as the coenzyme forms involved in flavin catalysis. Because the initial product of the vitamin biosynthetic pathway is unphosphorylated riboflavin (despite the fact that the early reaction steps involve intermediates carrying a position 5′-phosphate residue), the phosphorylation of riboflavin is a necessary step in all organisms, irrespective of the biosynthetic origin of their vitamin B_2 supply.

The presence of riboflavin kinase (Fig. 1, step **X**) in plant extracts has been reported in the 1950s and 1960s, but no detailed studies were conducted (Giri et al., 1957; Mitsuda et al., 1963). Sequence comparisons based on plant genome projects have recently uncovered the gene for riboflavin kinase from *A. thaliana*, tomato and rice.

The biochemical properties of the recombinant plant enzyme are similar to orthologous riboflavin kinases from *S. pombe* and to riboflavin kinase domains from bacteria. Structures of riboflavin kinases from *S. pombe* and *Homo sapiens* have recently been obtained by X-ray crystallography and have identified the protein as a member of a large kinase superfamily (Bauer et al., 2003; Karthikeyan et al., 2003). No FAD synthetase (Fig. 1, step **XI**) of plant origin has been described in any detail up to now.

Cellular location of the riboflavin pathway in plants

All plant genes involved in the biosynthesis of riboflavin, which have been described carry N-terminal extensions with a an approximate length of 63–120 amino acid residues as compared with the homologous microbial enzymes (Fischer et al., 2004b, 2005; Herz et al., 2000; Jordan et al., 1999). Although the catalytic domains are characterized by conservative evolution, even over very wide distances, as judged by sequence comparison, the N-terminal sequence extensions show little similarity. As an exception, the N-terminal sequences of riboflavin synthases from *Lycopersicon esculentum* and *Solanum tuberosum* show 91% identity, but the species are closely related. The closest similarity of the *A. thaliana* riboflavin synthase targeting sequence is to the N-terminal part of the orthologue from *Lotus japonicus* (26% identity and 18% similarity).

Computer analysis invariably interprets these N-terminal segments as targeting sequences. As shown above, truncated, pseudomature forms constructed after the computer prediction or sequence alignments with bacterial enzymes can be expressed in bacterial host strains where they yield catalytically active proteins. Studies with lumazine synthase from tomato provided direct evidence for plastid location (Jordan et al., 1999). In yeasts, the riboflavin biosynthetic enzymes are devoid of N-terminal extensions.

In contrast to enzymes involved in the biosynthesis of riboflavin in plants, there is no evidence for N-terminal sequence extensions in case of the riboflavin kinases of *A. thaliana*, tomato and rice. Thus, it appears likely that the kinase is located in the cytoplasm. In that case, riboflavin would have to be transported from the plastids into the cytoplasmic compartment, before conversion into the coenzyme form.

Evolution of the riboflavin pathway

During the past decade, hundreds of complete genomes have been reported. These data, together with information from incompletely sequenced genomes and from EST databases, enable the study of evolution processes at the level of protein-coding genes.

Surprisingly, the architecture of the *ribAB* gene of various plants follows the pattern of homologous eubacterial genes (as opposed to fungal or archael genes). In bifunctional *ribAB* genes, the GTP cyclohydrolase II domain is invariably at the C-terminal end of the fusion protein. This suggests that the fused gene has originated only once, unless we assume that there are unknown selection pressures that favour a C-terminal arrangement of the GTP cyclohydrolase II domain (Herz et al., 2000).

For the conversion of the GTP cyclohydrolase II product, **2**, into 5-amino-6-ribitylamino-2,4 (1*H*,3*H*)-pyrimidinedione (**6**), eubacteria, blue-green algae and higher plants use the pathway via the intermediate **3**, whereas fungi and archaea use a somewhat different pathway via **4**. Under these circumstances, it comes as no particular surprise that the plant deaminases show closer similarity with eubacterial than with fungal homologues (Fischer et al., 2004b). On the other hand, the plant riboflavin synthases are closely similar to those of yeasts (Fig. 10) (Fischer et al., 2005a).

Eubacteria typically specify bifunctional proteins with riboflavin kinase and FAD synthetase activities located in different domains. On the other hand, fungi and animals express riboflavin kinase and FAD synthetase as separate proteins. In this case, plants follow the same pattern as yeasts and animals.

It should be noted that two plant proteins, 5-amino-6-ribosylamino-2,4(1*H*,3*H*)-pyrimidinedione 5′-phosphate reductase (Fig. 1, step **III**) and FAD synthetase (Fig. 1, step **XI**), could not be identified by genome comparisons. Most probably, these plant proteins carry little or no similarity with the corresponding proteins from any of the other kingdoms.

Conclusions

Because flavocoenzymes are apparently indispensable in all organisms, they must be obtained by biosynthesis or from the environment. Riboflavin biosynthesis has been shown to proceed in plants and in autotrophic microorganisms. Contrariwise, the vitamin must be obtained from dietary sources by animals.

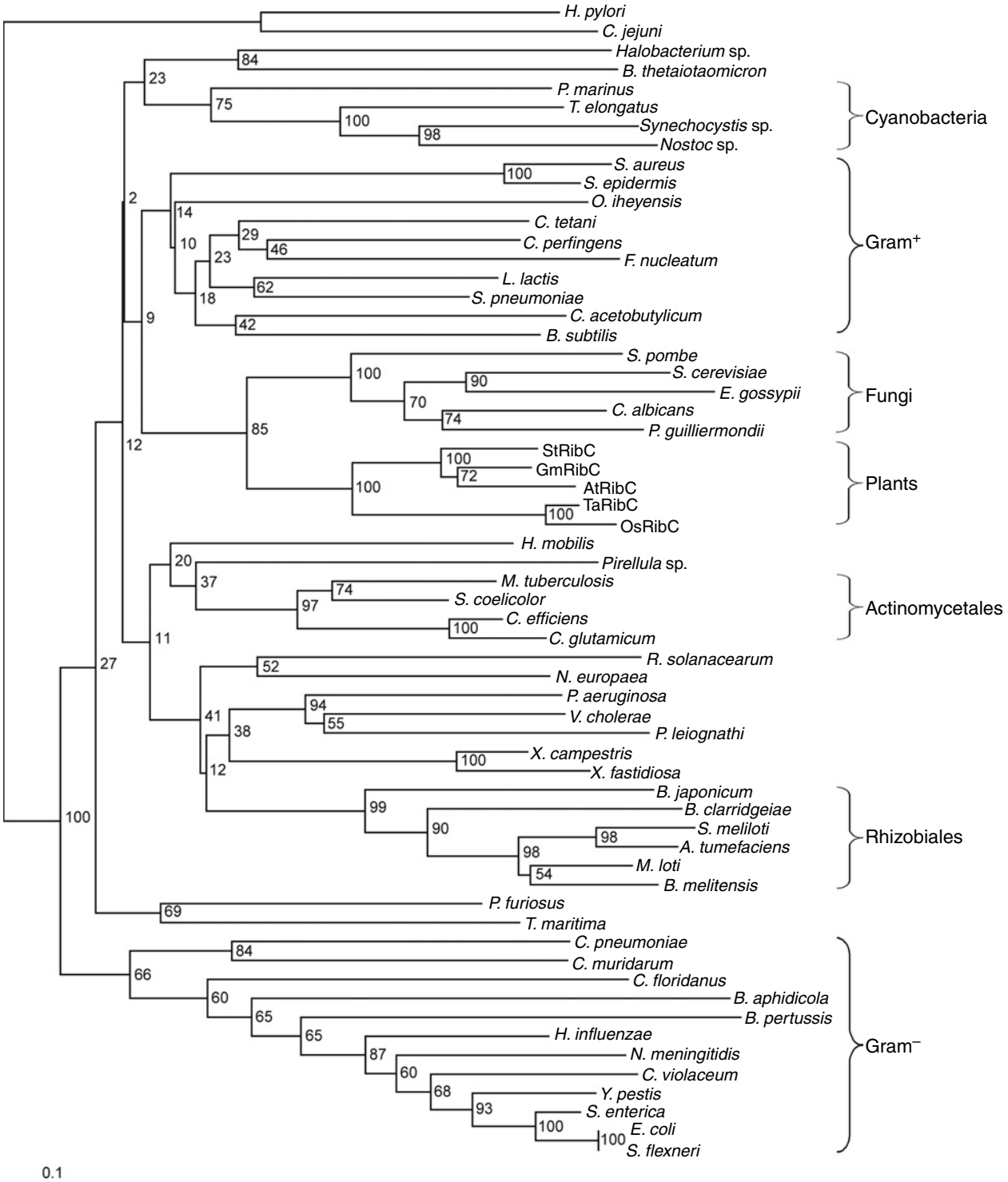

Fig. 10. Phylogenetic tree of riboflavin synthases from different organisms. The tree was deduced by neighbour-joining analysis based on the alignment of 61 riboflavin synthases. Gaps were removed from the alignment, and the total number of positions taken into account was 180. The numbers at the nodes are the statistical confidence estimates computed by the bootstrap procedure. The bar represents 0.1 PAM distance (Fischer et al., 2005a).

A bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase has been cloned and expressed from *A. thaliana*. The enzyme is remarkably similar to the bifunctional orthologues from eubacteria; more than that, both plant proteins resemble bacterial orthologues more closely than the yeast enzymes (Herz et al., 2000).

The similarity between the riboflavin pathway in eubacteria and plants is further emphasized by the presence of a plant deaminase that converts the product of GTP cyclohydrolase II as in eubacteria; fungi use a different sequence of reactions. Sequence comparison showed relatively close similarity between eubacterial and plant deaminases (Fischer et al., 2004b).

6,7-Dimethyl-8-ribityllumazine synthase from spinach has been cloned and expressed, and its structure has been determined by X-ray crystallography. The enzyme is a 532 symmetric icosahedral capsid consisting of 60 identical subunits (Jordan et al., 1999; Persson et al., 1999). That quaternary structure mimics the icosahedral lumazine synthase found in most eubacteria (with the exception, at the present state of information, of *Brucella abortus* and *Mycobacterium tuberculosis* (Braden et al., 2000; Morgunova et al., 2005; Zylberman et al., 2004), whereas yeasts and fungi form c_5-symmetric, pentameric lumazine synthases.

Riboflavin synthase has been partially purified from spinach (Mitsuda et al., 1970). Thirty years later, the cognate gene of *A. thaliana* has been cloned and expressed (Bacher and Eberhardt, 2001; Fischer et al., 2005a). The enzyme was shown to be a homotrimer similar to orthologous proteins from eubacteria and fungi. However, the purified *A. thaliana* riboflavin synthase was characterised by a bright yellow colour that was caused by non-covalent binding of riboflavin. In contrast, most other riboflavin synthases studied so far were isolated without bound riboflavin, indicating significantly lower affinity (Fischer et al., 2005a).

All riboflavin pathway enzymes from plants have N-terminal extensions that have been interpreted as targeting peptides, indicating that the biosynthesis of riboflavin proceeds in compartments. The sequences of the catalytic domains of each group of plant enzymes are closely similar, whereas the targeting sequences are devoid of significant sequence similarity showing organism-dependent variabilities.

There is no evidence for an N-terminal targeting sequence in case of riboflavin kinase of *A. thaliana*, tomato and rice. Thus, it appears likely that the kinase is located in the cytoplasm.

Characteristic architectural features (sequences and structures) of most enzymes involved in the plant riboflavin pathway closely resemble those of eubacteria, whereas the similarities between plants and yeasts are quite low. Thus, riboflavin biosynthesis in plants proceeds by the same reaction steps as in eubacteria, whereas fungi use a partly different pathway.

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Edited by D. Van Der Straeten