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DARWIN REVIEW

Plant B vitamin pathways and their compartmentation: a guide for the perplexed

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Abstract

The B vitamins and the cofactors derived from them are essential for life. B vitamin synthesis in plants is consequently as crucial to plants themselves as it is to humans and animals, whose B vitamin nutrition depends largely on plants. The synthesis and salvage pathways for the seven plant B vitamins are now broadly known, but certain enzymes and many transporters have yet to be identified, and the subcellular locations of various reactions are unclear. Although very substantial, what is not known about plant B vitamin pathways is regrettably difficult to discern from the literature or from biochemical pathway databases. Nor do databases accurately represent all that is known about B vitamin pathways—above all their compartmentation—because the facts are scattered throughout the literature, and thus hard to piece together. These problems (i) deter discoveries because newcomers to B vitamins cannot see which mysteries still need solving; and (ii) impede metabolic reconstruction and modelling of B vitamin pathways because genes for reactions or transport steps are missing. This review therefore takes a fresh approach to capture current knowledge of B vitamin pathways in plants. The synthesis pathways, key salvage routes, and their subcellular compartmentation are surveyed in depth, and encoded in the SEED database (http://pubseed.theseed.org/seedviewer.cgi?page=PlantGateway) for *Arabidopsis* and maize. The review itself and the encoded pathways specifically identify enigmatic or missing reactions, enzymes, and transporters. The SEED-encoded B vitamin pathway collection is a publicly available, expertly curated, one-stop resource for metabolic reconstruction and modeling.

Key words: Biosynthesis, biotin, compartmentation, folate, niacin, pantothenate, pyridoxine, riboflavin, thiamin.

Introduction

The B vitamins are a set of eight water-soluble enzyme cofactors and their derivatives: thiamin, riboflavin, niacin, pantothenate, pyridoxine, biotin, folate, and cobalamin. Their metabolic essentiality has long been recognized, as has the value of plants in human and animal diets as sources of all B vitamins except cobalamin (which higher plants lack). However, despite the nutritional significance of plant B vitamins (Fitzpatrick *et al*., 2012), and their cardinal roles in plants themselves (Smith *et al*., 2007), their biosynthetic routes are still not fully clear. This is partly because the low abundance of the pathway enzymes and transporters hampers classical biochemistry.

The advent of sequenced genomes revealed much conservation between B vitamin pathways in plants and bacteria or fungi, and many plant B vitamin synthesis enzymes were quickly cloned by homology and characterized as recombinant proteins (Roje, 2007). Such homology-driven research inevitably leaves components 'missing' from pathways when plants do things differently from other organisms (e.g. Tanabe *et al*., 2011) or when

the components are not identified in any organism (Hanson and Gregory, 2010). Some B vitamin enzymes and transporters have been experimentally localized within plant cells, not always reliably (e.g. Pinon *et al*., 2005; Tanabe *et al*., 2011); others have only predicted locations.

Plant B vitamin pathways are thus patchworks of well-lit areas where much is known, and darker ones that cloak missing steps, enzymes, and transporters, and uncertain compartmentation. Unfortunately, the complexity of the pathways and the scattered literature make it hard for non-specialist researchers, database annotators, and metabolic modellers to tell light areas from dark ones. The resulting shadowland is bad for all B vitamin research, and especially bad for efforts to build next-generation metabolic models that integrate B vitamin pathways (Seaver *et al*., 2012).

The above problems are basically those of all plant metabolic pathways, writ large. This review therefore explores a new *modus operandi* to transmit deep knowledge about metabolic pathways in the post-genomic era. The time-honoured way—specialist, digitally disconnected reviews that dwell mainly on past progress (i.e. on what is known)—is no longer adequate. It is now vital to make information seamlessly available in digital form and to define clearly what is unknown. If researchers and annotators can simply click to find exactly what is not known about a metabolic pathway, their creativity instantly becomes available to help solve the mysteries.

Our review captures knowledge of B vitamin synthesis in the model plants *Arabidopsis* and maize, and encodes it in a public database (SEED). For easy access, each B vitamin is covered in a stand-alone module consisting of a text section, a pathway diagram, a Supplementary table available at *JXB* online, and matching SEED resources (see Box 1). Both the review and the SEED pathways show which steps, enzymes, transporters, and locations remain unknown or enigmatic, and point out other mysteries. The overall aim is to spare researchers as much perplexity as possible. Accordingly, the review's title respectfully echoes that of a seminal philosophical and theological text by the 12th century rabbi Moses Maimonides (Maimonides, 1904). Following his example, text sections are short, normative consensus interpretations for practical use, for example in building compartmented pathway models. They are not discursive in tone, and do not present all the reasoning behind interpretations or all the relevant literature; fuller reasoning and literature coverage are given in the Supplementary tables.

Excellent conventional reviews of biosynthesis of plant B vitamins can be found in two recent volumes of *Advances in Botanical Research* (Rébeillé and Douce, 2011a, 2011b).

Riboflavin (vitamin B_2)

http://pubseed.theseed.org/SubsysEditor.cgi?page=ShowDiagram&subsystem=Riboflavin%2C_FMN_and_FAD_biosynthesis_in_plants

Overview of known steps and their compartmentation

Riboflavin is the precursor of the cofactors FMN and FAD, which are used by many oxidoreductases from the cytosol, plastids, mitochondria, and peroxisomes. Riboflavin synthesis takes place solely in plastids, from the precursors GTP and D-ribulose 5-phosphate (Roje, 2007; Fischer and Bacher, 2011) (Fig. 1). Riboflavin can be converted successively to FMN and FAD within plastids themselves, in the cytosol, or in mitochondria. There are also opposite pathways in which FAD and FMN are hydrolysed back to riboflavin (Roje, 2007).

Several riboflavin pathway enzymes are fused to each other or to domains of unknown function (Fig. 1, inset). Thus, the enzymes that initiate both branches of the synthesis pathway (GTPCH2 and DHBPS in Fig. 1) are fused, as are cytosolic riboflavin kinase and FMN hydrolase, which catalyse opposing reactions; the latter arrangement is discussed further below. The enzymes for the deamination and reduction steps in the pathway (PyrD and PyrR in Fig. 1) also seem at first sight to be fused, as in the *Escherichia coli* enzyme RibD; the reality is more complex (see below).

Missing enzymes

Two riboflavin synthesis enzymes are missing. One is the phosphatase PyrP; this enzyme is probably substrate specific as it evidently does not attack the upstream phosphorylated intermediates. The other missing enzyme is the reductase PyrR. As just noted, *E. coli* RibD is a fusion protein, with deaminase and reductase domains. *Arabidopsis* and other plants have two distinct RibD homologues. One has deaminase but not reductase activity (Fischer *et al*., 2004); the other is inferred to have reductase activity (Chatwell *et al*., 2006; Chen *et al*., 2006; Ouyang *et al*., 2010), but this is unproven. Several enzymes that interconvert riboflavin, FMN, and FAD are also missing. These include organellar riboflavin kinases, mitochondrial FMN adenylyltransferase, and extraplastidial FAD pyrophosphatases.

Missing transporters

The localization of the pathway enzymes implies that there are riboflavin transporters in plastids and mitochondria, and riboflavin uptake has been demonstrated for both organelles (Sandoval *et al*., 2008; Giancaspero *et al*., 2009). There must also be at least one riboflavin transporter in the plasma membrane because plants can absorb and secrete riboflavin (Stocks and Miller, 1965; Higa *et al*., 2008). As knocking out cytosolic riboflavin kinase or FAD synthetase is lethal (S. Roje, personal communication), mitochondria and plastids probably cannot export FMN or FAD *in vivo*.

Unresolved compartmentation issues

As peroxisomes contain flavoenzymes (Goyer *et al*., 2004; Wang *et al*., 2004; Ono *et al*., 2012) but appear unable to make FMN or FAD, they presumably import either the flavins themselves (as shown in Fig. 1) or flavinated holoenzymes. Peroxisomes are known to import mature, folded proteins (Walton *et al*., 1995).

Other unsolved mysteries

Major mysteries in the riboflavin pathway are the roles of domains fused to two of its enzymes (Fig. 1, inset). The putative reductase PyrR is fused to a C-terminal COG3236 (IPR012816) domain; this domain is also fused to the first enzyme of riboflavin synthesis

Box 1 Organization of the review

(i) Text sections. These summarize what is known about synthesis pathways and salvage pathways that mesh closely with synthesis, and highlight missing enzymes, transporters, and other mysteries ('missing' means that a protein or gene is inferred to exist but is not identified).

(ii) Pathway diagrams. These are formatted similarly to KEGG pathways. Compounds are shown as circles or ovals, enzymes and transporters as boxes, and enzymatic or transport reactions as arrows. Thick arrows are reactions that have been demonstrated directly or can be deduced from experimental evidence. Thin arrows are expected reactions; dotted arrows are more hypothetical reactions. Enzymes and transporters are colour coded as follows: white, gene(s) identified in plants; yellow, enigmatic in some way in plants; pale pink, gene missing in plants only; dark pink, gene missing in all organisms; crossed out in blue, strongly inferred to be absent. When reliable experimental evidence is available, reactions are assigned to the cytosol, plastid, mitochondrion, or peroxisome. Absent such evidence, localization is predicted bioinformatically for *Arabidopsis*.

Gene fusion events. Fusions of pathway enzymes to each other or to domains of known or unknown function are shown in an inset. Pathway enzymes that are fused together are coloured (other than grey) and joined by double lines; all other pathway enzymes are light grey. Fused domains of known function are solid red boxes; fused domains of unknown function are boxes containing a red 'X', and are denoted when possible by COG (Clusters of Orthologous Groups) and InterPro identifiers.

Disjunct enzymes. 'Disjunct enzymes' boxes contain plant proteins that cannot yet be definitely connected to pathways because the precise reaction catalysed or the subcellular location is unknown; some have homologues in other organisms that are implicated in B vitamin metabolism by experimental or bioinformatic data. Disjunct enzyme boxes are non-exhaustive; they include only the most striking cases.

(iii) Supplementary pathway tables. Each table outlines the evidence supporting the corresponding pathway diagram, gives a detailed bibliography (as PubMed IDs), and lists the *Arabidopsis* and maize genes for the pathway enzymes and transporters.

(iv) SEED resources. Each plant B vitamin pathway is encoded in the SEED database as a 'subsystem' (Overbeek *et al*., 2005) comprising the enzymes and transporters ('functional roles') in the pathway in *Arabidopsis* and maize. Subsystems are spreadsheets with columns of functional roles, rows of genomes, and cells populated by the genes implementing each functional role in each genome. The subsystems can be accessed via the links from the text section titles, Supplementary Tables S1–S7 at *JXB* online, or from the SEED Plant Gateway (http://pubseed.theseed.org/seedviewer.cgi?page=PlantGateway). As more plant genomes and pathways are added to SEED, the subsystems will be extended to include them.

The SEED also contains the above pathway diagrams, which are linked to (i) the functional roles (hover cursor over boxes); (ii) the underlying plant genomes (follow links to the corresponding gene/protein pages in SEED, TAIR, or MaizeSequence.org); and (iii) chemical compounds and reactions in KEGG (point cursor to circles and click). Each diagram can display which functional roles do not yet have associated genes in a given genome (i.e. which genes are missing). To use this feature, follow the directions on each diagram page to 'colour diagram by genome': the enzymes and transporters known to be encoded in the selected genome will then be highlighted green (indicating they are present), whereas missing genes will not change colour. Although protein compartmentation is encoded only for *Arabidopsis*, the data can probably be projected onto maize with a fair degree of confidence. Note also that, besides the above plant-specific resources, SEED contains hundreds of microbial genomes and subsystems covering many metabolic and other functions in microbes.

(GTPCH2) in certain bacteria (e.g. *Vibrio* spp., *Moritella* sp.) and occurs as a stand-alone protein in many others. The putative cytosolic FMN adenylyltransferase is fused to a C-terminal COG1058 (IPR001453) domain. Mammalian FMN adenylyltransferase likewise has a COG1058 domain, but this domain is N-terminal. COG1058 domains also occur in proteins involved in molybdopterin cofactor synthesis, in fusions with bacterial nicotinamide mononucleotide deaminases (Galeazzi *et al*., 2011), and as stand-alone proteins in various bacteria and fungi. A lesser mystery is the *in vivo* function of a HAD hydrolase (COG1011) domain fused to cytosolic riboflavin kinase. This domain has high FMN hydrolase activity *in vitro* (Sandoval and Roje, 2005) but as this activity reverses the riboflavin kinase reaction the fusion protein would seemingly catalyse a futile cycle.

Thiamin (vitamin B_1)

http://pubseed.theseed.org/SubsysEditor.cgi?page=ShowDiagram&subsystem=Thiamin_biosynthesis_in_plants

Overview of known steps and their compartmentation

Thiamin, in its active form thiamin diphosphate (ThDP), is the cofactor for transketolases, decarboxylases, and other enzymes that make or break C–C bonds. Thiamin contains pyrimidine and thiazole moieties, which are synthesized separately in plastids and then coupled together in plastids to give thiamin monophosphate (ThMP) (Goyer, 2010; Rapala-Kozik, 2011) (Fig. 2). The next steps are (in unknown order) export from the plastid and hydrolysis to thiamin (Goyer, 2010). Thiamin then is converted to ThDP in the cytosol (Fig. 2). A remarkable feature of the pathway is that the thiazole synthesis protein Thi4 uses itself as sulphur donor for thiazole formation and undergoes only a single turnover (Chatterjee *et al*., 2011). This makes thiazole energetically very costly to produce, and puts a high premium on salvaging thiazole moieties from ThDP breakdown (see below).

The biosynthetic enzymes hydroxymethylpyrimidine phosphate kinase and thiamine-phosphate pyrophosphorylase are fused together (Fig. 2, inset).

Missing enzymes

One prominent case is the phosphatase that converts ThMP to thiamin; however, this enzyme may not be a specific one (Rapala-Kozik *et al*., 2009; Goyer, 2010). A more subtly missing enzyme is a hydrolase to mediate the deadenylation step in thiazole synthesis (French *et al*., 2011). Enzymes that synthesize and hydrolyse thiamin triphosphate (which occurs in plants; Makarchikov *et al*., 2003) are missing.

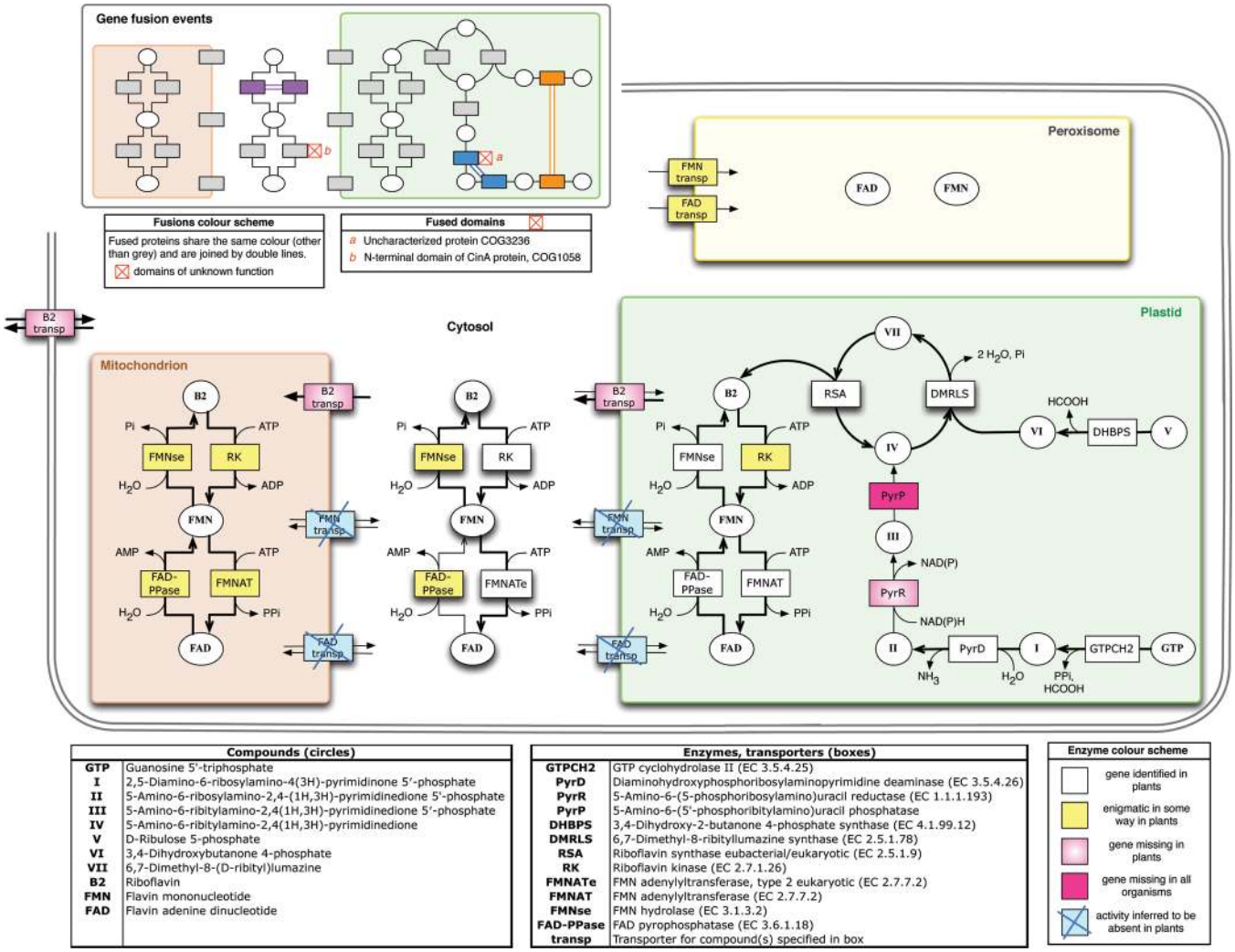


Fig. 1. Pathways of riboflavin synthesis and conversion to FMN and FAD. The inset depicts gene fusion events. Evidence supporting the reactions and transport steps shown is summarized in Supplementary Table S1 at *JXB* online. Symbols and colours are explained in Box 1.

Missing transporters

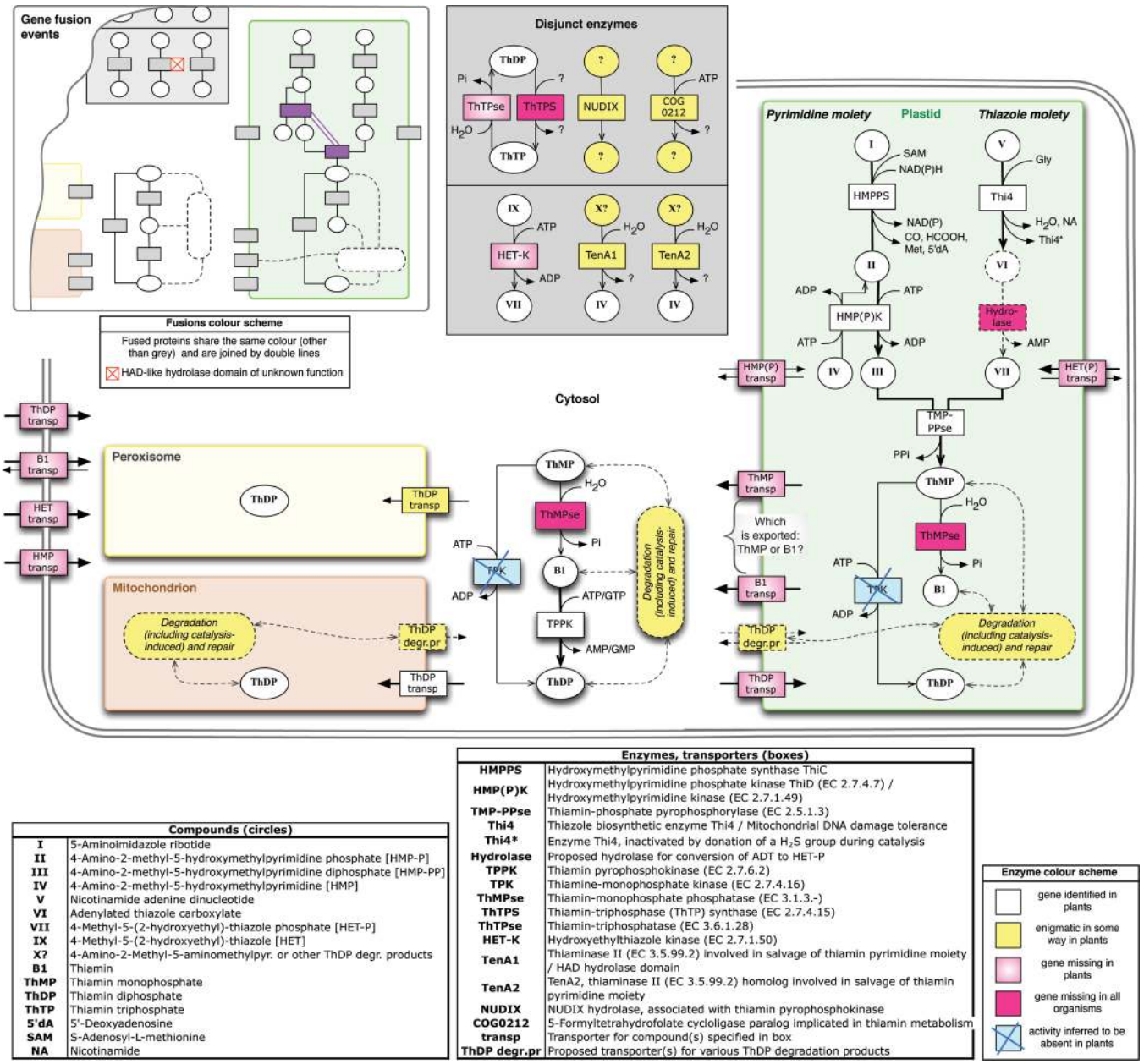
Since ThDP is made in the cytosol, plastids must export ThMP or thiamin (Fig. 2). As plastids and mitochondria (and probably peroxisomes) contain ThDP-dependent enzymes (Foulon *et al*., 1999; Goyer, 2010), these organelles all presumably import ThDP from the cytosol, but plant ThDP carriers are so far known only for mitochondria (Frelin *et al*., 2012). The rescue of thiamin mutants by thiamin or its pyrimidine or thiazole moieties (Feenstra, 1964) implies that there are plasma membrane transporters for all of these, and plastidial transporters for pyrimidine and thiazole. Organelles may also transport thiamin breakdown products (see below).

Unresolved compartmentation issues

It is not known whether ThMP is hydrolysed in plastids or cytosol, and thus whether plastids export thiamin or ThMP. Nothing is known about the subcellular distribution of thiamin and its phosphates or about levels of free and enzyme-bound ThDP.

Other unsolved mysteries

Major mysteries concern the salvage of ThDP, which decomposes spontaneously (Pribat *et al*., 2011) and is destroyed during catalysis (McCourt *et al*., 2006). Salvage enzymes for the pyrimidine and thiazole moieties are known in microbes, and two such enzymes—TenA (pyrimidine salvage) and ThiM (thiazole salvage)—have plant homologues. The activities and locations of these homologues are unknown or unclear. Plants also have homologues of the putative salvage enzyme COG0212 (Pribat *et al*., 2011). Furthermore, plants have strong, free-standing homologues of a Nudix hydrolase that is fused in *Schizosaccharomyces pombe* to thiamin pyrophosphokinase, the enzyme that converts thiamin to ThDP. The function of this Nudix protein is not known, but may relate to thiamin salvage. A minor mystery is whether plants make adenosine thiamin triphosphate, as do bacteria and animals. This compound was detected in roots (Bettendorff *et al*., 2007) but could have come from the microflora. Lastly, genetic evidence (Ajjawi *et al*., 2007) largely, but not wholly, excludes there being a plastidial ThMP kinase. ThMP



Compounds (circles)	
I	5-Aminoimidazole ribotide
II	4-Amino-2-methyl-5-hydroxymethylpyrimidine phosphate [HMP-P]
III	4-Amino-2-methyl-5-hydroxymethylpyrimidine diphosphate [HMP-PP]
IV	4-Amino-2-methyl-5-hydroxymethylpyrimidine [HMP]
V	Nicotinamide adenine dinucleotide
VI	Adenylated thiazole carboxylate
VII	4-Methyl-5-(2-hydroxyethyl)-thiazole phosphate [HET-P]
IX	4-Methyl-5-(2-hydroxyethyl)-thiazole [HET]
X?	4-Amino-2-Methyl-5-aminomethylpyr. or other ThDP degr. products
B1	Thiamin
ThMP	Thiamin monophosphate
ThDP	Thiamin diphosphate
ThTP	Thiamin triphosphate
5'dA	5'-Deoxyadenosine
SAM	S-Adenosyl-L-methionine
NA	Nicotinamide

Enzymes, transporters (boxes)	
HMPPS	Hydroxymethylpyrimidine phosphate synthase ThiC
HMP(P)K	Hydroxymethylpyrimidine phosphate kinase ThiD (EC 2.7.4.7) / Hydroxymethylpyrimidine kinase (EC 2.7.1.49)
TMP-PPse	Thiamin-phosphate pyrophosphorylase (EC 2.5.1.3)
Thi4	Thiazole biosynthetic enzyme Thi4 / Mitochondrial DNA damage tolerance
Thi4*	Enzyme Thi4, inactivated by donation of a H_2S group during catalysis
Hydrolase	Proposed hydrolase for conversion of ADT to HET-P
TPPK	Thiamin pyrophosphokinase (EC 2.7.6.2)
TPK	Thiamine-monophosphate kinase (EC 2.7.4.16)
ThMPse	Thiamin-monophosphate phosphatase (EC 3.1.3.-)
ThTPS	Thiamin-triphosphase (ThTP) synthase (EC 2.7.4.15)
ThTPse	Thiamin-triphosphatase (EC 3.6.1.28)
HET-K	Hydroxyethylthiazole kinase (EC 2.7.1.50)
TenA1	Thiaminase II (EC 3.5.99.2) involved in salvage of thiamin pyrimidine moiety / HAD hydrolase domain
TenA2	TenA2, thiaminase II (EC 3.5.99.2) homolog involved in salvage of thiamin pyrimidine moiety
NUDIX	NUDIX hydrolase, associated with thiamin pyrophosphokinase
COG0212	5-Formyltetrahydrofolate cycloligase paralog implicated in thiamin metabolism
transp	Transporter for compound(s) specified in box
ThDP degr.pr	Proposed transporter(s) for various ThDP degradation products

Fig. 2. Pathways of thiamin synthesis and salvage. Insets depict gene fusion events and disjunct enzymes. Evidence supporting the reactions and transport steps shown is summarized in Supplementary Table S2 at *JXB* online. Symbols and colours are explained in Box 1. Note the uncertainty surrounding the nature, sites of formation, intracellular transport, and fate of thiamin degradation products.

kinase occurs in bacteria (Jurgenson *et al*., 2009); its presence in plastids would make plastids autonomous for ThDP synthesis.

Pyridoxine (vitamin B_6)

http://pubseed.theseed.org/SubsysEditor.cgi?page=ShowDiagram&subsystem=Pyridoxine_%28vitamin_B6%29_biosynthesis_in_plants

Overview of known steps and their compartmentation

The active form of vitamin B_6, pyridoxal 5'-phosphate (PLP), is the cofactor for numerous enzymes that mainly act on amino acids. PLP is synthesized *de novo* in the cytosol by the two-protein (PDX1/PDX2) PLP synthase complex from pentose phosphate, triose phosphate, and glutamine precursors (Fitzpatrick, 2011) (Fig. 3). PLP can also be made in chloroplasts or cytosol from pyridoxamine 5'-phosphate (PMP) or pyridoxine 5'-phosphate (PNP) by the salvage enzyme pyridoxamine 5'-phosphate oxidase (Fitzpatrick, 2011) (Fig. 3).

Side activities of PLP-dependent enzymes can convert PLP to PMP (Mason *et al*., 1969) and PMP to PLP (Leoncini *et al*., 1998). PLP, PMP, and PNP can be dephosphorylated to pyridoxal, pyridoxamine, and pyridoxine, respectively, which can be rephosphorylated by pyridoxal kinase in the cytosol or plastids. Pyridoxal is reduced by a chloroplastic pyridoxal reductase to pyridoxine (Herrero *et al*.,

2011), which can be glucosylated by a UDP-glucose-dependent glucosyltransferase. Pyridoxine glucosides are major forms of vitamin B_6 in plants (Gregory and Ink, 1987).

Missing enzymes

Neither the phosphatase(s) that hydrolyse PLP, PMP, and PNP nor the glucosyltransferase(s) that form pyridoxine glucosides (Tadera *et al*., 1982) have been identified, and their subcellular locations are unknown.

Missing transporters

Plant tissues take up pyridoxine (Titiz *et al*., 2006), and some form of B_6 is transferred from maternal tissues to embryos (Tambasco-Studart *et al*., 2005), indicating transport in both directions across the plasma membrane. Organellar transport of PLP (or derivatives convertible to PLP) is also indicated because (i) plastids, mitochondria, and peroxisomes contain PLP-dependent enzymes that can lose the cofactor (Mason *et al*., 1969); (ii) the activity of these enzymes thus depends on an intraorganellar PLP pool; and (iii) this pool must ultimately come from *de novo* synthesis in the cytosol. Nothing is known about these transport steps; they are presumably carrier mediated as in other organisms (Said *et al*., 2003; Stolz and Vielreicher, 2003).

Unresolved compartmentation issues

It is not known where in the cell the (often large) pyridoxine glucoside pool is located or how other vitamin B_6 forms are distributed among subcellular compartments, although pyridoxal and pyridoxine have been found in plastids (Havaux *et al*., 2009).

Other unsolved mysteries

One mystery is that plant PPOX has an N-terminal domain (COG0062, IPR004443) homologous to NAD(P)H hydrate epimerases (see below) of other organisms (Marbaix *et al*.,

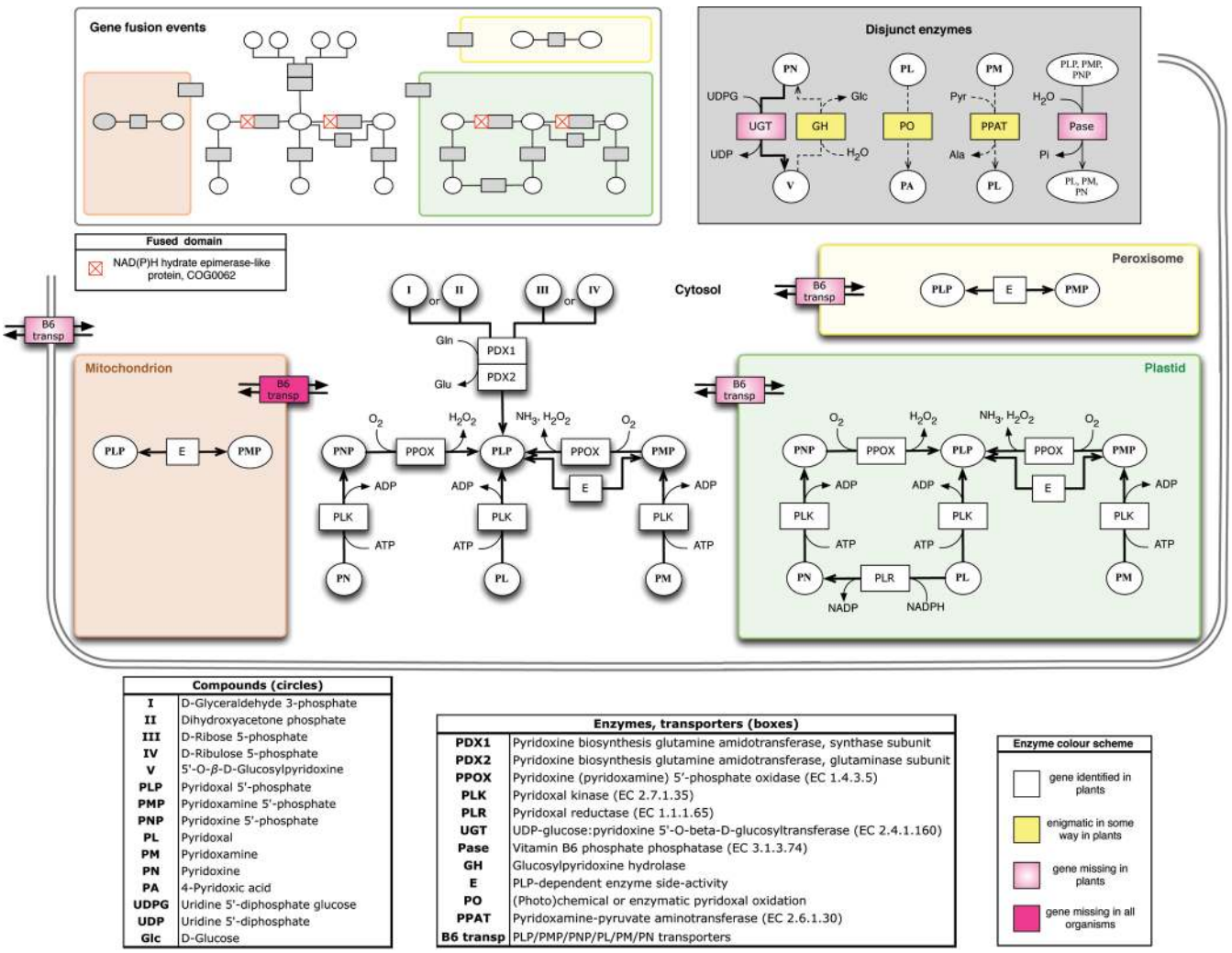


Fig. 3. Pathways of pyridoxine synthesis and salvage. Insets depict gene fusion events and disjunct enzymes. Evidence supporting the reactions and transport steps shown is summarized in Supplementary Table S3 at *JXB* online. Symbols and colours are explained in Box 1.

2011); the function of this domain is unknown (Sang *et al*., 2011). Secondly, it is not known whether plants have a specific pyridoxamine-pyruvate aminotransferase (Huang *et al*., 2011) or whether this is a side activity of other aminotransferases (Wada and Snell, 1962). Thirdly, the origin and fate of 4-pyridoxic acid in plants (Sampson *et al*., 1995; Raschke *et al*., 2011) are unknown. In other organisms, 4-pyridoxic acid is either a damage product of pyridoxal formed by (photo) chemical oxidation or by promiscuous aldehyde oxidases and dehydrogenases (Reiber, 1972; Stanulović *et al*., 1976), or an intermediate in pyridoxal degradation (Yokochi *et al*., 2006). Lastly, it is not known whether, or where, pyridoxine glucosides are hydrolysed.

Niacin (vitamin B_3)

http://pubseed.theseed.org/SubsysEditor.cgi?page=ShowDiagram&subsystem=Niacin%2C_NAD_and_NADP_biosynthesis_in_plants

Overview of known steps and their compartmentation

The active forms of niacin (a term for nicotinate and nicotinamide) are nicotinamide adenine dinucleotide (NAD) and its 2'-phosphate ester (NADP), which are coenzymes in hundreds of redox reactions. NAD is also the substrate for sirtuins, poly(ADP-ribose) polymerases, and other enzymes that release the nicotinamide moiety. The *de novo* synthesis pathway begins in the plastid, where nicotinate mononucleotide (NaMN) is formed from aspartate, dihydroxyacetone phosphate, and a ribose phosphate moiety. NaMN then exits to the cytosol, where successive adenylation and amidation give nicotinate adenine dinucleotide (NaAD) and then NAD (Noctor *et al*., 2011) (Fig. 4). The adenylation, and probably amidation, steps also occur in mitochondria (Di Martino and Pallotta, 2011). NAD is converted to NADP in cytosol, plastids, peroxisomes, and probably mitochondria (Di Martino and Pallotta, 2011; Noctor *et al*., 2011) (Fig. 4).

The nicotinamide released by NAD-consuming enzymes is salvaged by hydrolysis to nicotinate, which is converted to NaMN that re-enters the *de novo* pathway. NAD is cleaved by various enzymes to nicotinamide mononucleotide (NMN), which can be reconverted directly to NAD or hydrolysed to nicotinamide and recycled as above. These salvage reactions are all cytosolic; the NMN→NAD reaction is also probably mitochondrial (Di Martino and Pallotta, 2011; Noctor *et al*., 2011) (Fig. 4).

Quinolinate synthase has a catalytically essential 4Fe–4S cluster and is fused to a SufE domain that participates in formation of the cluster (Murthy *et al*., 2007). Plastidial NAD kinase is fused to a calmodulin-binding domain (Turner *et al*., 2004).

Missing enzymes

No plant genes are known for NMN nucleosidase, which hydrolyses NMN to nicotinamide (Wagner *et al*., 1986), or mitochondrial NMN/NaMN adenylyltransferase, which converts NMN to NAD and NaMN to NaAD (Di Martino and Pallotta, 2011). Other cases for which plant genes are unknown include NADP phosphatase (Roje *et al*., 1999), the enzymes that form *N*-glucosyl (Taguchi *et al*., 1997) and *N*-methyl (Upmeier *et al*., 1988) derivatives of nicotinate, and the enzyme that demethylates *N*-methylnicotinate (trigonelline) to nicotinate (Shimizu and Mazzafera, 2000).

Missing transporters

A minimal set of transporter proteins (Fig. 4) has been identified that, based on their activities in heterologous systems or when reconstituted in proteoliposomes, can account for intracellular traffic in NAD and its metabolites. However, only the peroxisomal NAD transporter has genetic support for the proposed function *in vivo* (Bernhardt *et al*., 2012) so that the physiological importance of the others is unclear. If they are not important *in vivo*, further transporters await discovery (Noctor *et al*., 2011).

Unresolved compartmentation issues

The subcellular locations of NADP phosphatase, nicotinate glucosyltranferase and methylase, and trigonelline demethylase are unknown. The cytosolic compartmentation of some salvage enzymes is hypothetical (inferred from lack of targeting signals); certain of these enzymes are also probably in mitochondria as noted above.

Other unsolved mysteries

Hydration of the NAD(P)H pyridine ring occurs spontaneously and as a side activity of glyceraldehyde 3-phosphate dehydrogenase, giving mixed *S* and *R* forms of NAD(P)H hydrate that inhibit certain NAD(P)-linked enzymes (Marbaix *et al*., 2011). Bacteria, yeast, and mammals are known to recycle both forms to NAD(P)H via the sequential action of an epimerase and a dehydratase specific for the *S* form (Marbaix *et al*., 2011). Plants have homologues of both enzymes. One mystery, noted above, is that the epimerase homologue is fused to the B_6 salvage enzyme PPOX—which has no evident rationale. Another mystery is compartmentation: the epimerase and dehydratase are known or predicted to be plastidial, but hydrate formation is almost surely not confined to plastids. Hydrate recycling may thus demand intercompartmental transport of the hydrates or multiple targeting of the recycling enzymes.

Folate (vitamin B_9)

http://pubseed.theseed.org/SubsysEditor.cgi?page=ShowDiagram&subsystem=Folate_biosynthesis_in_plants

Overview of known steps and their compartmentation

Tetrahydrofolate (THF) acts as a carrier for one-carbon units and, less often, as an electron donor. THF contains pterin, *p*-aminobenzoate, and glutamate moieties. The pterin is made in the

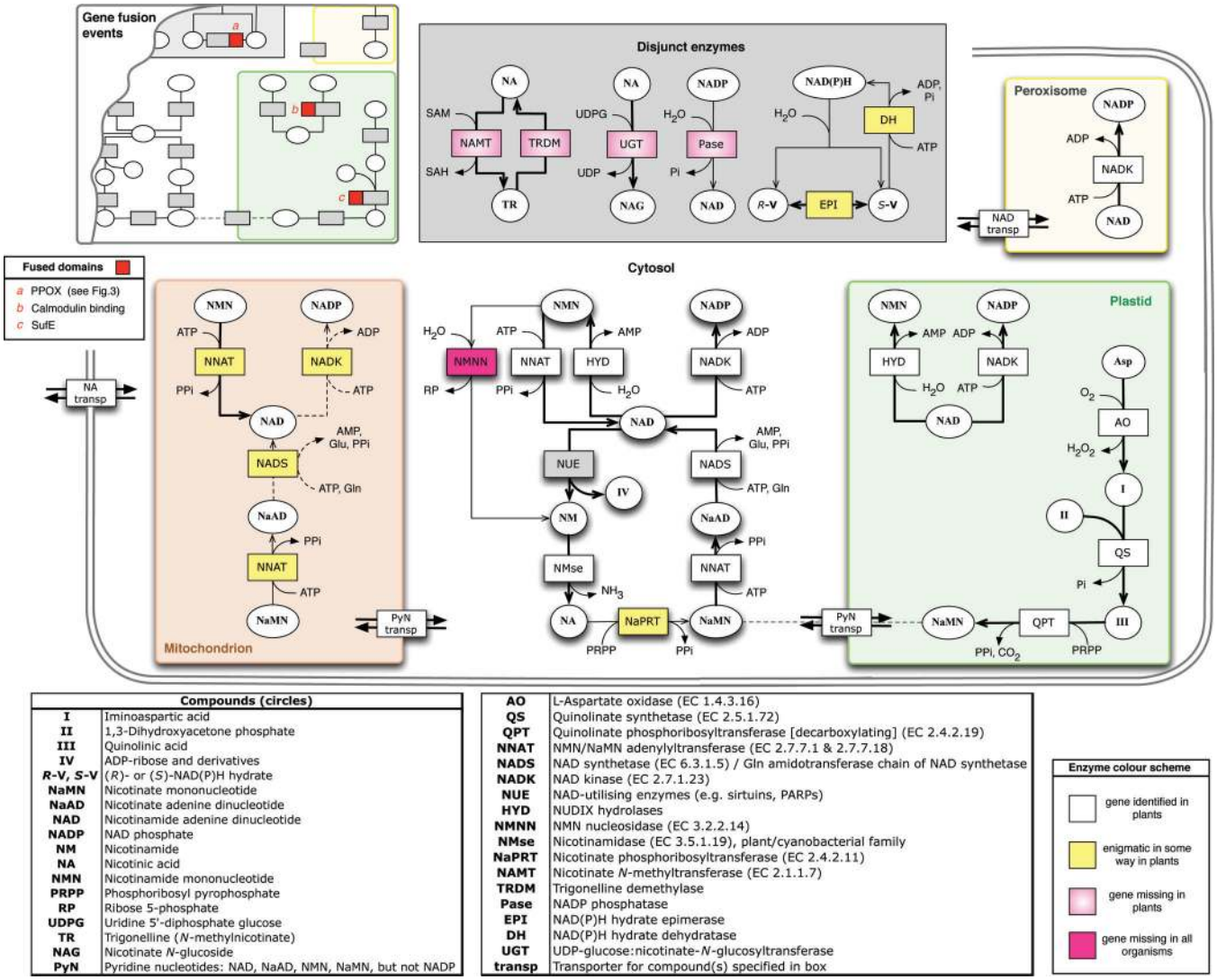


Fig. 4. Pathways of niacin and pyridine nucleotide synthesis and salvage. NAD(P) and NAD(P)H hydrolysis by Nudix hydrolases probably occurs in multiple compartments and is shown for simplicity only in the cytosol. Insets depict gene fusion events and disjunct enzymes. Evidence supporting the reactions and transport steps shown is summarized in Supplementary Table S4 at *JXB* online. Symbols and colours are explained in Box 1. The light grey dashed line traces the connection between mitochondrial and cytosolic parts of the synthesis pathway.

cytosol from GTP, *p*-aminobenzoate is made from chorismate in plastids, and the two are coupled together in mitochondria to give dihydropteroate; glutamylation and reduction steps in mitochondria then yield THF (Hanson and Gregory, 2011; Ravanel *et al*., 2011) (Fig. 5). THF and its one-carbon derivatives (collectively termed folates) occur mainly as polyglutamates; these have a short γ-linked polyglutamyl tail that can be added in mitochondria, plastids, or the cytosol (Fig. 5). Folate polyglutamates can be imported into vacuoles, where the polyglutamyl tail can be removed by γ-glutamylhydrolase. *p*-Aminobenzoate can be converted to its glucose ester in the cytosol, and imported into vacuoles.

The two enzymes that activate the pterin and couple it to *p*-aminobenzoate (HPPK and DHPS in Fig. 5) are fused, and the enzyme for the reduction step (dihydrofolate reductase) is fused to thymidylate synthase (which forms dihydrofolate).

Missing enzymes

The enzyme that dephosphorylates the pterin synthesis intermediate dihydroneopterin monophosphate and the corresponding gene have not been identified in any organism. Non-specific phosphatase(s) may be responsible (Suzuki and Brown, 1974). Pterins can be converted to β-D-glycosides (Díaz de la Garza *et al*., 2004); nothing is known about the sugar moiety or about the enzyme(s) and gene(s) involved.

Missing transporters

Taken collectively, data on the subcellular distribution of folates and folate-dependent enzymes, and on uptake and metabolism of folates and their precursors, strongly imply the existence of nine carrier-mediated transport steps: six for

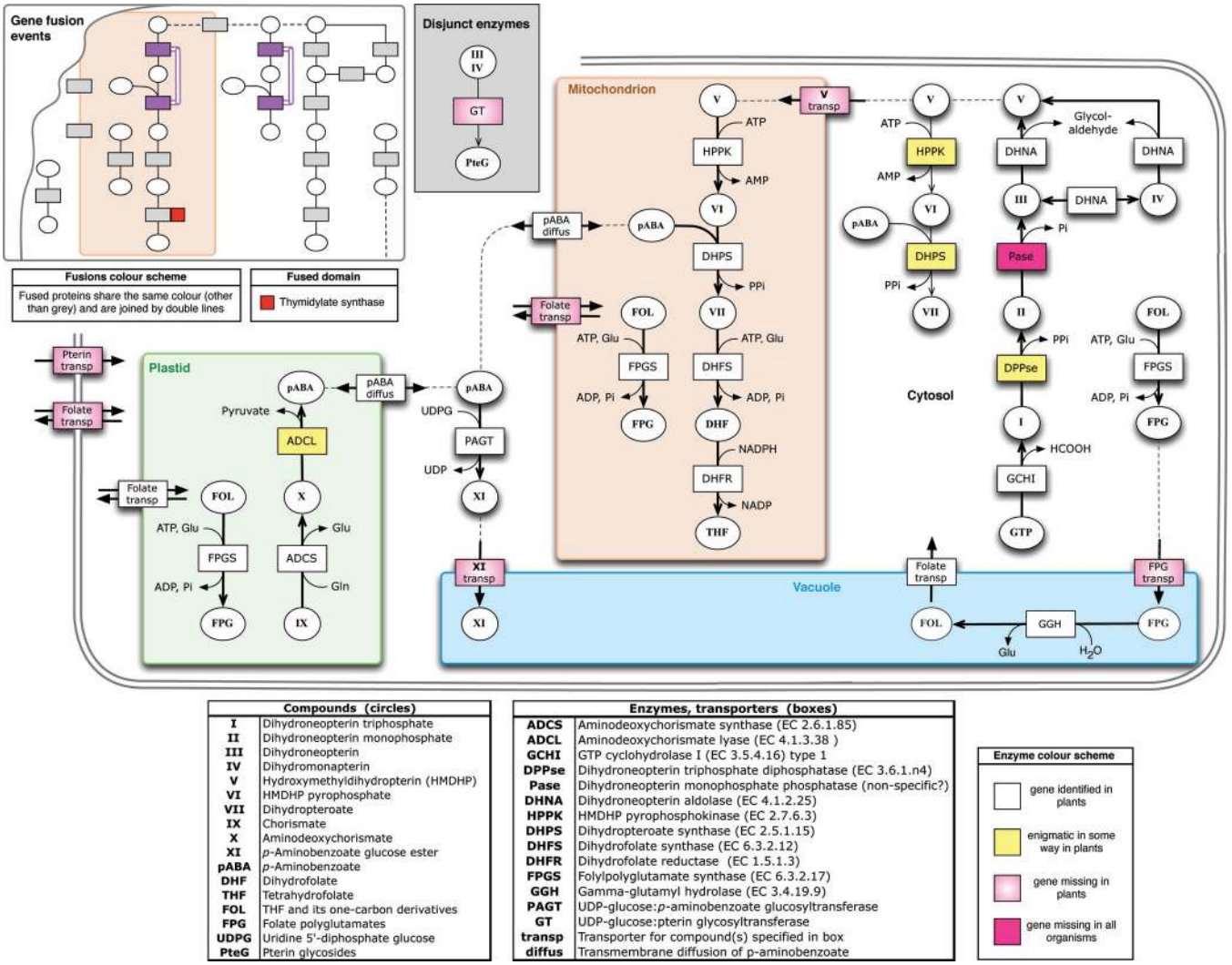


Fig. 5. Pathways of folate synthesis. Insets depict gene fusion events and a disjunct enzyme. Evidence supporting the reactions and transport steps shown is summarized in Supplementary Table S5 at *JXB* online. Symbols and colours are explained in Box 1. Light grey dashed lines trace connections between the same metabolite in various reactions or transport steps.

folates or folate polyglutamates, two for pterins, and one for *p*-aminobenzoate glucose ester (Fig. 5) (Hanson and Gregory, 2011). Genes have been identified for only two of these nine steps, namely plastidial and vacuolar folate transport (Hanson and Gregory, 2011).

Unresolved compartmentation issues

In *Arabidopsis*, a second copy of the gene for the fusion enzyme HPPK–DHPS specifies a cytosolic protein (Storozhenko *et al*., 2007). The metabolic significance of this enzyme is unclear, given its disconnection from subsequent pathway steps in the mitochondrion; its ablation or overexpression had no impact on folate levels (Navarrete *et al*., 2012). It is not known whether any other plants have a cytosolic HPPK–DHPS. *Arabidopsis* has three genes that specify cytosolic, mitochondrial, and plastidial isoforms of the enzyme that adds polyglutamate tails (FPGS in Fig. 5) (Ravanel *et al*., 2001); it is not clear whether the plastidial enzyme is also cross-targeted to mitochondria and vice versa (Mehrshahi *et al*., 2010). As other plants may have fewer than three FPGS genes (Akhtar *et al*., 2008) multiple targeting of FPGS could be common.

Other unsolved mysteries

A Nudix family hydrolase able to mediate the depyrophosphorylation step in pterin synthesis (DPPse in Fig. 5) has been identified but it is non-specific and its major *in vivo* role may be to eliminate oxidized nucleotides (Klaus *et al*., 2005; Yoshimura *et al*., 2007). The true DPPse may thus remain to be found. The enzyme thought to catalyse step two in *p*-aminobenzoate synthesis (ADCL in Fig. 5) belongs to the branched-chain aminotransferase family and has high D-amino acid aminotransferase activity (Basset *et al*., 2004; Funakoshi *et al*., 2008). The role of ADCL in *p*-aminobenzoate synthesis and D-amino acid metabolism has not been tested genetically.

Biotin (vitamin B_8)

http://pubseed.theseed.org/SubsysEditor.cgi?page=ShowDiagram&subsystem=Biotin_biosynthesis_in_plants

Overview of known steps and their compartmentation

Biotin is attached to a specific lysine residue in four carboxylases that occur in the cytosol, mitochondria, and plastids, and to seed biotin-binding proteins (Nikolau *et al*., 2003). Biotin is made from pimeloyl-CoA in four steps (Alban, 2011), of which the first is peroxisomal (Tanabe *et al*., 2011); the other three steps are almost surely mitochondrial (Fig. 6). The enzymes for steps two and three, DAPA synthase and dethiobiotin synthase, can occur as a fusion protein (Fig. 6, inset). The ligase that attaches biotin to carboxylases is present in cytosol, plastids, and mitochondria (Fig. 6).

Missing enzymes

The source of pimeloyl-CoA is not known in plants, but appears to differ from those in bacteria (Lin and Cronan, 2011). The first enzyme of biotin synthesis, KAPA synthase, is peroxisomal in *Arabidopsis* and *Aspergillus oryzae* (Tanabe *et al*., 2011), and biotin synthesis in *Aspergillus nidulans* requires β-oxidation (Magliano *et al*., 2011). Pimeloyl-CoA in plants and fungi may thus come from fatty acid degradation via the concerted action of β-, α-, and ω-oxidation enzymes in peroxisomes.

Seed biotin-binding proteins are probably biotinylated by a special enzyme, not by the ligases that act on biotin-dependent enzymes, because (i) their biotinylated lysine residue is in a motif unlike that recognized by regular biotin–protein ligases; (ii) *Arabidopsis* and *E. coli* ligases cannot biotinylate the apoprotein form of biotin-binding proteins; and (iii) soybean biotin-binding protein expressed in *E. coli* is not biotinylated (Job *et al*., 2001). Biotin-binding proteins may act as biotin reserves; if so, a biotinidase is required to cleave the biotin–lysine bond (Alban *et al*., 2000).

Missing transporters

As the biotin pathway is split between peroxisomes and mitochondria, and biotin-dependent enzymes occur in mitochondria,

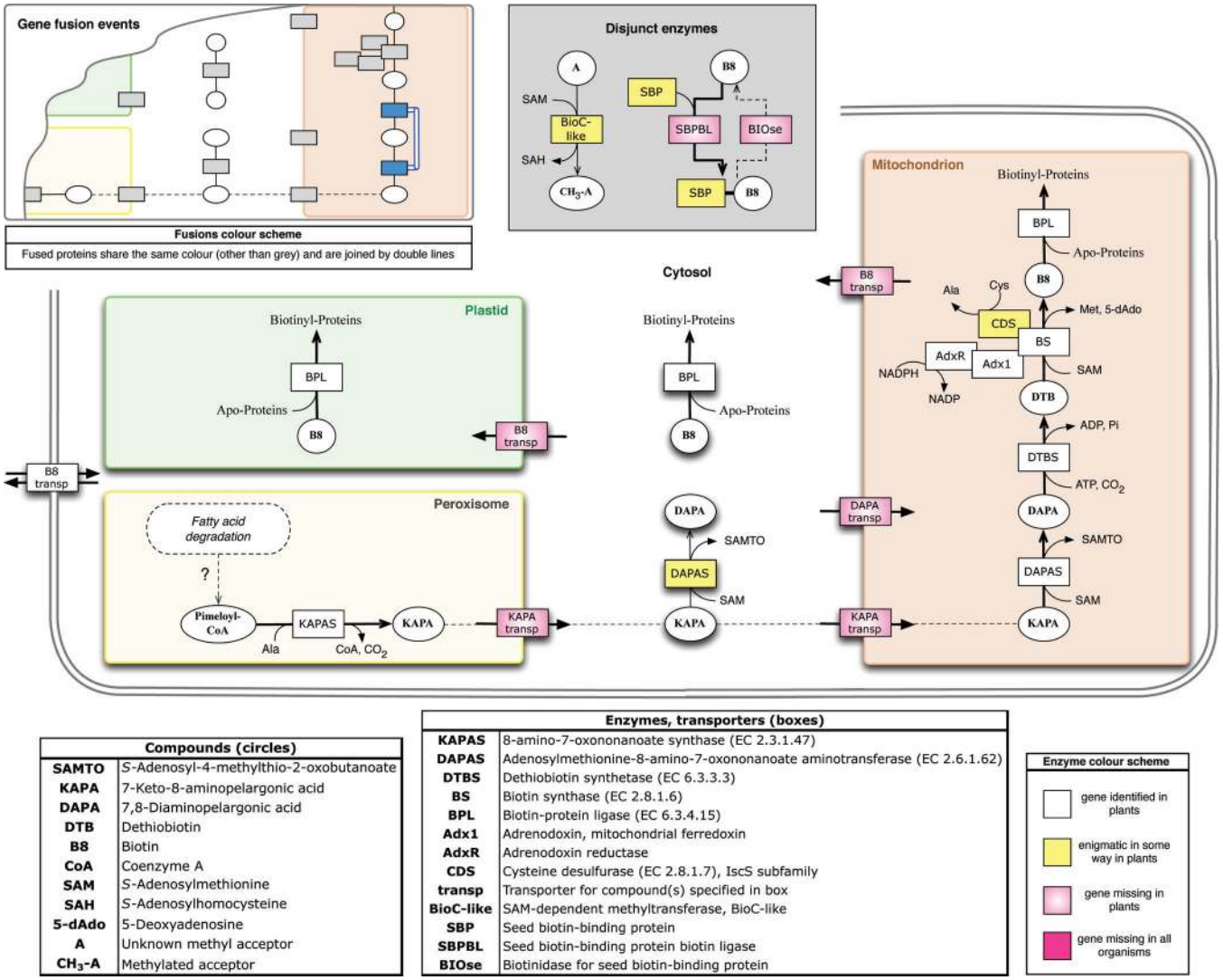


Fig. 6. Pathways of biotin synthesis. The insets depict gene fusion events and disjunct enzymes. Evidence supporting the reactions and transport steps shown is summarized in Supplementary Table S6 at *JXB* online. Symbols and colours are explained in Box 1. The light grey dashed line traces the connection between peroxisomal and mitochondrial parts of the synthesis pathway.

cytosol, and plastids, transporters must be invoked for KAPA export from peroxisomes and import into mitochondria, and for biotin export from mitochondria and import into plastids (Fig. 6). Also, cytosolically expressed *E. coli* DAPA synthase can functionally replace the native, mitochondrial enzyme in *Arabidopsis* (Patton *et al*., 1996), implying mitochondrial DAPA import.

Unresolved compartmentation issues

DAPA synthase and dethiobiotin synthase are encoded by adjacent genes that can be alternatively spliced to specify a bifunctional fusion protein or separate enzymes (Muralla *et al*., 2008). The fusion and the separate dethiobiotin synthase are predicted to be mitochondrial and the separate DAPA synthase is predicted to be cytosolic; experimental data on the occurrence and targeting of the separate proteins are lacking.

Arabidopsis has two genes for biotin–protein ligase. HCS1 is essential and appears to specify cytosolic, plastidial, and mitochondrial isoforms of this enzyme; HCS2 is dispensable and probably inactive (Denis *et al*., 2002; Puyaubert *et al*., 2008). It is not clear where HCS2 proteins are targeted or if they have any function *in vivo*.

Other unsolved mysteries

Sulphur insertion into dethiobiotin by biotin synthase is enigmatic in plants. In bacteria, the sulphur comes from an iron–sulphur cluster on the synthase itself and a flavodoxin electron transfer chain is required (Fugate and Jarrett, 2012). In *Arabidopsis*, mitochondrial adrenodoxin, adrenodoxin reductase, and cysteine desulphurase are essential accessory components of the biotin synthase reaction (Picciocchi *et al*., 2003). Plants have homologues of the BioC methyltransferase from the *E. coli* pimeloyl synthesis pathway; the *Arabidopsis* homologue did not complement an *E. coli* Δ*bioC* strain (O. Frelin, unpublished), supporting the view that plants and bacteria differ in pimeloyl synthesis (see above). What plant BioC-like proteins do is thus a mystery.

Pantothenate (vitamin B_5)

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Overview of known steps and their compartmentation

Pantothenate is the precursor of coenzyme A (CoA), the acyl carrier in scores of reactions. Pantothenate is made in the cytosol from pantoate and β-alanine, then converted to CoA in five steps, all probably cytosolic (Webb and Smith, 2011); the fifth step may also occur in plastids and peroxisomes (Fig. 7). The three-step pathway to pantoate is not fully defined, but it is clear that step two, conversion of α-ketoisovalerate to ketopantoate, is mitochondrial (Ottenhof *et al*., 2004). Step one, α-ketoisovalerate formation from valine, is thus probably catalysed by a mitochondrial branched-chain aminotransferase (Taylor *et al*., 2004). The enzyme for step three (ketopantoate reduction to pantoate) is not known, so it is unclear whether this step is located in mitochondria or the cytosol.

Missing enzymes

Although the plant enzyme that reduces ketopantoate is unknown (Webb and Smith, 2011), mitochondrial acetohydroxy acid isomeroreductase is a good candidate. In certain bacteria, acetohydroxy acid isomeroreductase (IlvC) doubles as ketopantoate reductase (Merkamm *et al*., 2003), and plant acetohydroxy acid isomeroreductase reduces ketopantoate efficiently *in vitro* (Dumas *et al*., 1995). This enzyme occurs in plant mitochondria as well as in plastids, its main location (Heazlewood *et al*., 2004).

Missing transporters

Depending on where ketopantoate is reduced, mitochondria must export either pantoate or ketopantoate to the cytosol, probably via a carrier (Fig. 7). No carrier for pantoate or ketopantoate has been identified in any organism and, being weak acids, these compounds could conceivably just diffuse across membranes. Such diffusion may explain pantoyl lactone uptake (Rathinasabapathi and Raman, 2005).

Supplied pantothenate or pantethine (the oxidized form of pantetheine) can correct pantothenate or CoA synthesis lesions (Rubio *et al*., 2006; Jonczyk *et al*., 2008), implying that there are plasma membrane transporters for both precursors. A dual-function transporter seems unlikely inasmuch as the *E. coli* pantothenate transporter PanF does not transport pantethine (Vallari and Rock, 1985; Balibar *et al*., 2011).

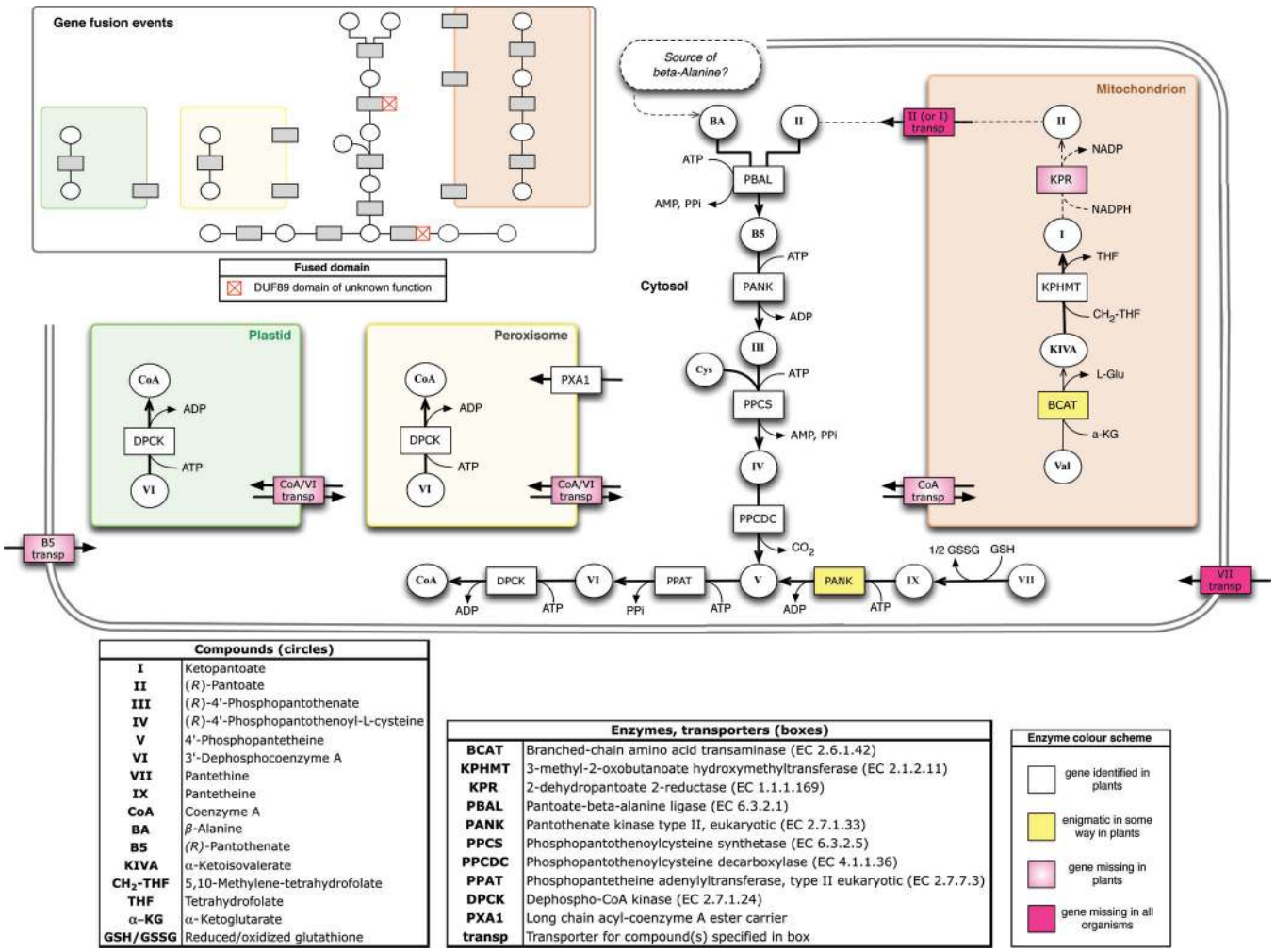
As CoA is made in the cytosol and CoA-dependent enzymes occur in peroxisomes, plastids, and mitochondria, these organelles all need CoA transporters (Webb and Smith, 2011). The only known plant CoA carrier is a peroxisomal one that imports acyl-CoA esters, not CoA itself (Kanai *et al*., 2010). There is presumably also a peroxisomal carrier to return CoA released by acyl-CoA oxidation to the cytosol.

Unresolved compartmentation issues

Proteomic data suggest that the enzyme that converts dephospho-CoA to CoA (DPCK in Fig. 7) occurs in plastids and peroxisomes as well as the cytosol (Reumann *et al*., 2009; Ferro *et al*., 2010). If it does, the missing peroxisomal and plastidial carriers (see above) might transport dephospho-CoA instead of, or in addition to, CoA.

Other unsolved mysteries

The salvage route from pantethine to CoA has not been characterized but probably involves non-enzymatic reduction by glutathione to give pantetheine (Durr and Cortas, 1964) and then phosphorylation to give phosphopantetheine (Fig. 7). The phosphorylation may be mediated by pantothenate kinase, as in some bacteria (Ward *et al*., 1955; Balibar *et al*., 2011). The source of β-alanine in plants is unknown and may not be unitary; possibilities include the metabolism of spermine, propionate, and uracil, but not the decarboxylation of aspartate (Rathinasabapathi *et al*., 2000; Raman and Rathinasabapathi, 2004; Webb and Smith, 2011). Finally, plants have two pantothenate kinases, one of which has a C-terminal DUF89 (COG1578) domain of unknown



Compounds (circles)	
I	Ketopantoate
II	(*R*)-Pantoate
III	(*R*)-4'-Phosphopantothenate
IV	(*R*)-4'-Phosphopantothenoyl-L-cysteine
V	4'-Phosphopantetheine
VI	3'-Dephosphocoenzyme A
VII	Pantethine
IX	Pantetheine
CoA	Coenzyme A
BA	*β*-Alanine
B5	(*R*)-Pantothenate
KIVA	α-Ketoisovalerate
CH_2-THF	5,10-Methylene-tetrahydrofolate
THF	Tetrahydrofolate
α-KG	α-Ketoglutarate
GSH/GSSG	Reduced/oxidized glutathione

Enzymes, transporters (boxes)	
BCAT	Branched-chain amino acid transaminase (EC 2.6.1.42)
KPHMT	3-methyl-2-oxobutanoate hydroxymethyltransferase (EC 2.1.2.11)
KPR	2-dehydropantoate 2-reductase (EC 1.1.1.169)
PBAL	Pantoate-beta-alanine ligase (EC 6.3.2.1)
PANK	Pantothenate kinase type II, eukaryotic (EC 2.7.1.33)
PPCS	Phosphopantothenoylcysteine synthetase (EC 6.3.2.5)
PPCDC	Phosphopantothenoylcysteine decarboxylase (EC 4.1.1.36)
PPAT	Phosphopantetheine adenylyltransferase, type II eukaryotic (EC 2.7.7.3)
DPCK	Dephospho-CoA kinase (EC 2.7.1.24)
PXA1	Long chain acyl-coenzyme A ester carrier
transp	Transporter for compound(s) specified in box

Enzyme colour scheme
gene identified in plants
enigmatic in some way in plants
gene missing in plants
gene missing in all organisms

Fig. 7. Pathways of pantothenate and CoA synthesis. Ketopantoate reduction could in principle be localized in the mitochondrion or the cytosol, but is shown only in the mitochondrion for simplicity and because this is the likelier possibility. The inset depicts a gene fusion event. Evidence supporting the reactions and transport steps shown is summarized in Supplementary Table S7 at *JXB* online. Symbols and colours are explained in Box 1. The light grey dashed line traces the connection between mitochondrial and cytosolic parts of the synthesis pathway.

function that is conserved in one of the mammalian pantothenate kinase isoforms (Tilton *et al*., 2006). DUF89 homologues also occur as stand-alone proteins and as fusions to HAD hydrolases, fructose-1,6-bisphosphatase, or other enzymes, and have a metal-binding site (Bitto *et al*., 2005; Tilton *et al*., 2006).

Four final generalizations

The pathway diagrams (Figs 1–7) highlight four recurrent features of B vitamin pathways, namely (i) missing transporters; (ii) missing hydrolases; (iii) enzymes not joined to mainline pathways; and (iv) fusions of pathway enzymes to domains of unknown or uncertain function. These are all areas in which discoveries are waiting to be made.

Missing transporters

Of 44 B-vitamin-related transporters that are known or strongly inferred to exist, 33 (75%) are missing; they come from all pathways except niacin. This contrasts sharply with the situation for biosynthetic enzymes where only six out of 52 (12%) are missing (of which some may be activities of known non-specific enzymes and so not strictly missing). The lopsided knowledge of B vitamin transporters versus enzymes (Fig. 8A) has parallels throughout plant metabolism (Linka and Weber, 2010).

Missing hydrolases

Almost all the genes missing from synthesis and salvage pathways encode enzymes from just three of the six Enzyme Classification (EC) classes: oxidoreductases, transferases, and hydrolases, particularly phosphatases (Fig. 8B). It is not coincidental that so many hydrolases are missing, as such enzymes typically belong to superfamilies (e.g. HAD and Nudix superfamilies) that have many members in plants and tend to have multiple substrates. These factors have deterred investigations (i) because there may be too many candidates to knock out or test for enzyme activity conveniently; and (ii) because of doubt that a single, specific enzyme truly exists.

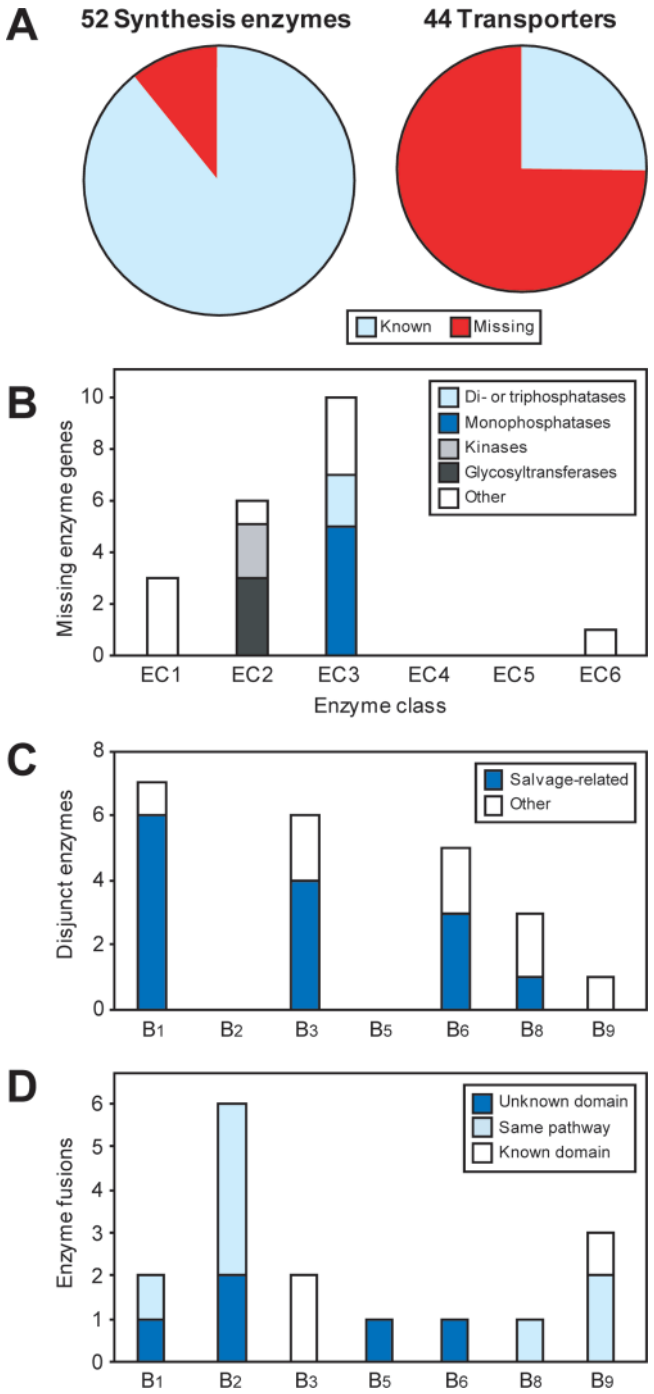


Fig. 8. Four recurrent features of B vitamin pathways. (A) The imbalance between missing biosynthetic enzymes and missing transporters. The areas of the circles are proportional to the total numbers of enzymes or transporters; the sizes of the red sectors represent the percentages that are missing. (B) The concentration of missing enzymes in three of the six EC enzyme classes, and the high frequency of missing hydrolases that cleave phosphate monoester or diester bonds. (C) The large number of disjunct enzymes, of which the majority are most probably related to salvage. (D) The prevalence of fusions between pathway enzymes and between pathway enzymes and other domains of known or unknown function. Vitamin abbreviations: B_1, thiamin; B_2, riboflavin; B_3, niacin; B_5, pantothenate; B_6, pyridoxine; B_8, biotin; B_9, folate.

Disjunct enzymes

The pathway diagrams include 22 enzymes that are linked to B vitamins by metabolic, biochemical, or comparative genomic evidence, but that cannot be properly fitted into a pathway for want of information on the precise reaction catalysed or on subcellular location. Although not an exhaustive inventory, these disjunct enzymes illustrate how numerous such enzymes are, and how almost two-thirds (14 out of 22) are known, or can reasonably be inferred, to mediate salvage of vitamins or their precursors (Fig. 8C). The latter statistic underscores how little is known about B vitamin salvage in plants (or other organisms), salvage pathways being characteristically dispensable and hence hard to dissect genetically. Thiamin has the highest number of disjunct enzymes imputed to salvage (six); this mirrors a particular lack of knowledge about thiamin breakdown, which is complex (Goyer, 2010; Pribat *et al*., 2011).

Fusions with unknown domains

Of the total of ~70 enzymes in the pathway diagrams whose genes are known, 16 (~20%) exist as some sort of fusion. This value far exceeds the typical incidences (≤3%) of multidomain fusions in eu- and prokaryotic proteomes (Enright *et al*., 1999; Salim *et al*., 2011) and the incidences for *Arabidopsis* and maize proteomes (~2%) that can be estimated using the Gene deFuser program (Salim *et al*., 2011). Eight of the 16 fusions are to enzymes in the same pathway, three are to other proteins of known function, and five are to domains of unknown function (Fig. 8D). Most fusions of the pathway enzymes among themselves and to other known proteins can be explained by a need to channel or sequester labile compounds, for example the fusions of riboflavin synthesis enzymes (whose substrates and products are unstable) and the dihydrofolate reductase–thymidylate synthase fusion in the folate synthesis pathway (dihydrofolate being the unstable product of thymidylate synthase). The fusions to unknown domains may well have a similar rationale. For instance, reactive compounds could arise via chemical or enzymatic damage to vitamins or their precursors, and unknown fusion domains could repair or recycle these damage products (Marbaix *et al*., 2011; Seaver *et al*., 2012).

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Enzymes and corresponding genes involved in riboflavin (B_2) metabolism in *Arabidopsis* (AT) and maize.

Table S2. Enzymes and the corresponding genes involved in thiamin (B_1) metabolism in *Arabidopsis* (AT) and maize.

Table S3. Enzymes and corresponding genes involved in pyridoxine (vitamin B_6) metabolism in *Arabidopsis* (AT) and maize.

Table S4. Enzymes and corresponding genes involved in niacin (vitamin B_3) metabolism in *Arabidopsis* (AT) and maize.

Table S5. Enzymes and corresponding genes involved in folate (vitamin B_9) metabolism in *Arabidopsis* (AT) and maize.

Table S6. Enzymes and corresponding genes involved in biotin (vitamin B_8) metabolism in *Arabidopsis* (AT) and maize.

Table S7. Enzymes and corresponding genes involved in pantothenate (vitamin B_5) and coenzyme A metabolism in *Arabidopsis* (AT) and maize.

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