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Evidence for folate-salvage reactions in plants

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Summary

Folates *in vivo* undergo oxidative cleavage, giving pterin and *p*-aminobenzoylglutamate (*p*ABAGlu) moieties. These breakdown products are excreted in animals, but their fate is unclear in microorganisms and unknown in plants. As indirect evidence from this and previous studies strongly suggests that plants can have high folate-breakdown rates (approximately 10% per day), salvage of the cleavage products seems likely. Four sets of observations support this possibility. First, cleavage products do not normally accumulate: pools of *p*ABAGlu (including its polyglutamyl forms) are equivalent to, at most, 4–14% of typical total folate pools in *Arabidopsis thaliana, Lycopersicon esculentum* and *Pisum sativum* tissues. Pools of the pterin oxidation end-product pterin-6-carboxylate are, likewise, fairly small (3–37%) relative to total folate pools. Second, little *p*ABAGlu built up in *A. thaliana* plantlets when net folate breakdown was induced by blocking folate synthesis with sulfanilamide. Third, *A. thaliana* and *L. esculentum* tissues readily converted supplied breakdown products to folate synthesis precursors: *p*ABAGlu was hydrolysed to *p*-aminobenzoate and glutamate, and dihydropterin-6-aldehyde was reduced to 6-hydroxymethyldihydropterin. Fourth, both these reactions were detected *in vitro*; the reduction used NADPH as cofactor. An alternative salvage route for *p*ABAGlu, direct reincorporation into dihydrofolate via the action of dihydropteroate synthase, appears implausible from the properties of this enzyme. We conclude that plants are excellent organisms in which to explore the biochemistry of folate salvage.

Keywords: *p*-aminobenzoylglutamate, pterins, folate salvage, Arabidopsis, tomato, pea.

Introduction

Tetrahydrofolate (THF) and its one-carbon (C_1) substituted derivatives – collectively termed folates – are vital cofactors for various C_1 transfer reactions. They are tripartite molecules consisting of pterin, *p*-aminobenzoate (*p*ABA), and glutamate moieties, and typically have a short, γ-linked polyglutamyl tail attached to the first glutamate (Figure 1a). Plants, bacteria and fungi synthesize folates *de novo* whereas higher animals need a dietary supply (Cossins, 2000; Green *et al.*, 1996).

Folates undergo spontaneous oxidative or photooxidative scission of the C^9–N^{10} bond at physiological pH to yield a pterin and *p*-aminobenzoylglutamate (*p*ABAGlu) or its polyglutamyl forms (pABAGlu$_n$) (Figure 1a) (Gregory, 1989). Such non-enzymatic cleavage is thought to be the main way folates break down in all organisms, although proteins may sometimes facilitate the reaction (Scott, 1984; Suh *et al.*, 2001). Folates vary in susceptibility to cleavage, THF and its oxidation product dihydrofolate (DHF) being among the most vulnerable (Gregory, 1989; Hillcoat *et al.*, 1967; Reed and Archer, 1980). For THF and DHF, the first pterins formed in the reaction are, respectively, tetrahydro- and dihydropterin-6-aldehyde; further oxidation can convert the tetrahydro to the dihydro form, and both to the fully oxidized, aromatic form (Hillcoat *et al.*, 1967; Reed and Archer, 1980; Whiteley *et al.*, 1968) (Figure 1a). Still further oxidation converts pterin-6-aldehyde to pterin-6-carboxylate and perhaps other end products (Lowry *et al.*, 1949).

The *p*ABAGlu and pterin moieties from folate breakdown are excreted in the urine in mammals (Scott, 1984). Nothing

is known about the fate of these products in plants, but fragmentary data from microorganisms suggest their re-use in folate synthesis (Figure 1b). Thus (i) enzymes that release *p*ABA from *p*ABAGlu are known from bacteria (Hussein *et al.*, 1998; Sherwood *et al.*, 1985); (ii) the protozoan parasite *Leishmania* and bacterial cell extracts use pterin-6-aldehyde for folate synthesis, suggesting the capacity to reduce both the pterin ring and the aldehyde side chain to give the folate synthesis intermediate 6-hydroxymethyldihydropterin (Figure 1a) (Brown *et al.*, 1961; Nare *et al.*, 1997; Shiota, 1959); and (iii) enzyme activities mediating these reductions are known in *Leishmania* and *Escherichia coli* (Bello *et al.*, 1994; Mitsuda and Suzuki, 1971). For *p*ABAGlu, another potential salvage route is via the folate-synthesis enzyme dihydropteroate synthase (DHPS), which can use *p*ABAGlu in place of *p*ABA, giving DHF. The DHPS enzymes of *E. coli* and *Plasmodium* have V_{max}/K_m values for *p*ABAGlu that are approximately 100-fold lower than for *p*ABA, suggesting that the *p*ABAGlu reaction is insignificant *in vivo* (Ferone, 1973; Swedberg *et al.*, 1979). However, a *Pneumococcus* DHPS shows far less preference for *p*ABA over *p*ABAGlu (Ortiz, 1970), and the *Lactobacillus* enzyme shows none (Shiota *et al.*, 1969), so in these cases salvage via DHPS may be important. However *p*ABAGlu is recycled, its polyglutamyl tail may first need to be removed by γ-glutamyl hydrolase (Figure 1b) (Orsomando *et al.*, 2005).

In mammals, whole-body folate-breakdown rates are normally approximately 0.5% per day (Gregory and Quinlivan, 2002). There are no such estimates for plants, but indirect evidence suggests much higher values. Thus post-harvest studies of leaves and fruits point to net breakdown rates of approximately 10% per day at 20°C (Scott *et al.*, 2000; Stålsjö *et al.*, 2003). Similarly, in *Arabidopsis thaliana* plantlets given the folate-synthesis inhibitor sulfanilamide (Prabhu *et al.*, 1998), the rate of decline in C_1 fluxes suggests a possible folate loss rate of ≥10% per day. If plants have high folate-breakdown rates, it is important to know whether – and how – they salvage the fragments. Accordingly, in this work we first demonstrated that breakdown rates can be high, then began an exploration of salvage reactions. The systems studied were Arabidopsis and pea (*Pisum sativum*) leaves, in which most biochemical work on plant folates has been done, and fruit of tomato (*Lycopersicon esculentum*), which is a target for folate metabolic engineering (Díaz de la Garza *et al.*, 2004).

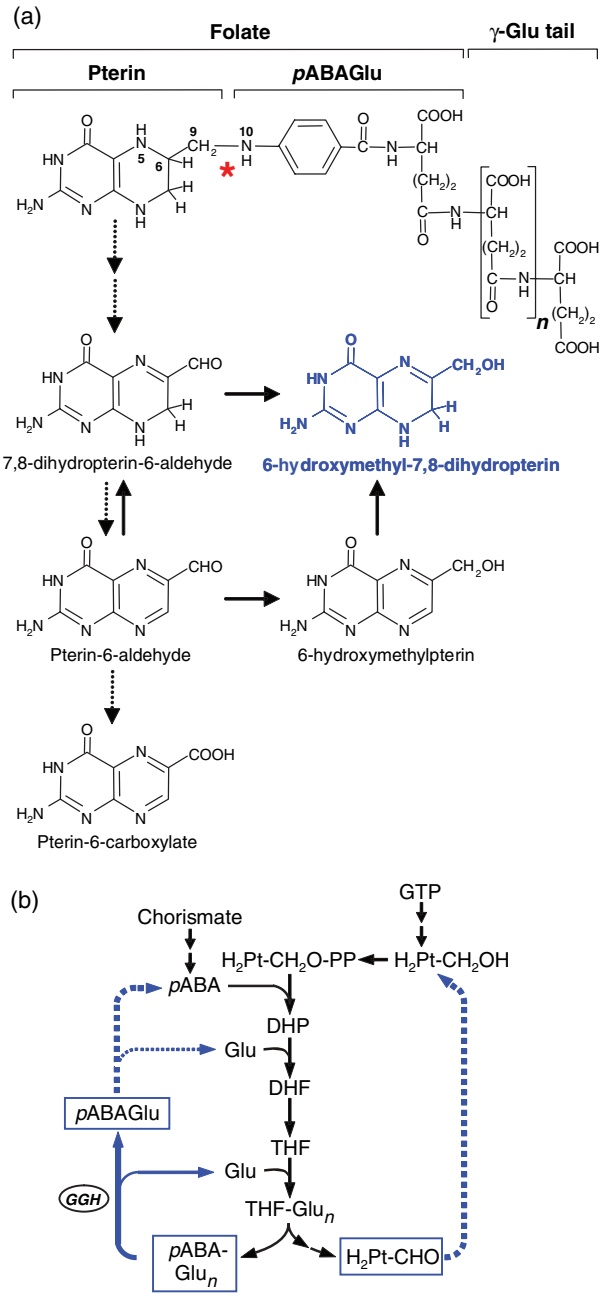

Figure 1. Folate structure, breakdown and potential salvage reactions.
(a) Structures and relationships of folates (the one shown is tetrahydrofolate, THF) and their breakdown products. Folates usually have a γ-linked polyglutamyl tail of up to about six residues attached to the first glutamate. C_1 units (e.g. formyl, methyl) can be coupled to the N^5 and/or N^{10} positions. Oxidative cleavage of the C^9–N^{10} bond (red asterisk) yields a pterin plus *p*ABAGlu or *p*ABAGlu$_n$. For THF, the pterin is 5,6,7,8-tetrahydropterin-6-aldehyde; this readily oxidizes to 7,8-dihydropterin-6-aldehyde (which is also formed directly by oxidative cleavage of dihydrofolate, DHF). 7,8-Dihydropterin-6-aldehyde can be further oxidized to pterin-6-aldehyde and pterin-6-carboxylate, or salvaged by reduction to the folate synthesis intermediate 6-hydroxymethyl-7,8-dihydropterin (blue). Pterin-6-aldehyde is also potentially convertible to 6-hydroxymethyl-7,8-dihydropterin (via reduction back to 7,8-dihydropterin-6-aldehyde, or reduction of the side chain to give 6-hydroxymethylpterin followed by ring reduction). Pterin-6-carboxylate seems not to be salvageable. Dotted arrows, (photo)chemical oxidations; solid arrows, enzymatic reductions.
(b) Folate-salvage reactions (blue arrows) in relation to folate biosynthesis. Dotted arrows denote reactions that are known in microorganisms but are hypothetical in plants. GGH, γ-glutamyl hydrolase; H_2Pt-CHO, dihydropterin-6-aldehyde; H_2Pt-CH_2OH, hydroxymethyl-7,8-dihydropterin; -PP, pyrophosphate; DHP, dihydropteroate.

Results

Folate-breakdown rates in Arabidopsis leaves and tomato fruit

For Arabidopsis, the folate-breakdown rate was investigated in liquid-cultured plants by blocking folate synthesis with sulfanilamide and monitoring the decline in leaf folate content (Figure 2a). Relative to the untreated control, total folate fell by 49% after 4 days' treatment.

For tomato, matched pairs of fruit were selected: one was detached at breaker stage and allowed to ripen, the other left on the plant. All fruit reached the red-ripe stage in 7 days, at which time the detached fruit had, on average, only 38% of the folate content of the attached fruit (Figure 2b). Assuming that folate contents of attached tomatoes change little between breaker and red-ripe stages (Basset *et al.*, 2002), it can be estimated that post-harvest breakdown was 62% in 7 days.

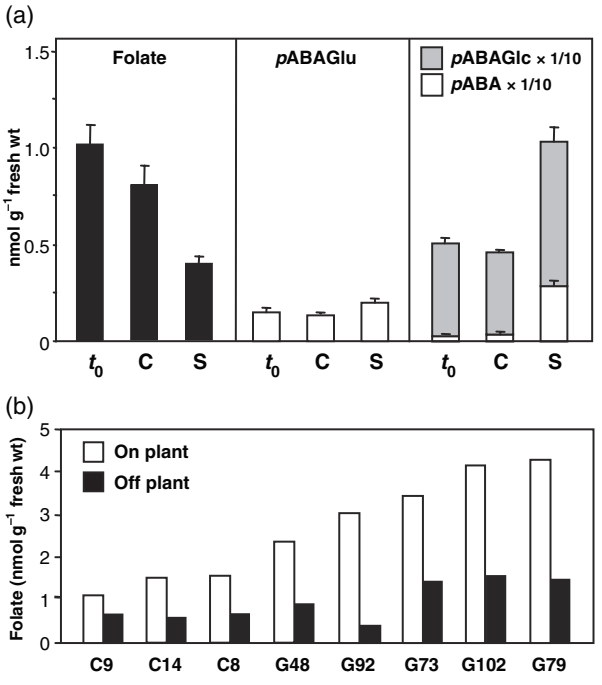

Figure 2. Evidence for substantial folate breakdown in plants.
(a) Folate loss in sulfanilamide-treated Arabidopsis plantlets. Total folate, total *p*ABAGlu (*p*ABAGlu plus *p*ABAGlu$_n$), free *p*ABA and *p*ABA glucose ester (*p*ABAGlc) levels in leaves of liquid-cultured plantlets before (t_0) and after incubation without (C) or with 2 mM sulfanilamide (S) for 4 days. Plantlets were 19 days old at t_0. Data are means of three replicates and SE. Note that the free *p*ABA and *p*ABA glucose ester values are multiplied ×1/10.
(b) Net folate breakdown in detached tomato fruit. Matched pairs of fruit were chosen from vector-alone control plants (C) or plants with elevated folate levels due to overexpression of the folate synthesis enzyme GTP cyclohydrolase I (G) (Díaz de la Garza *et al.*, 2004). At breaker stage, one fruit of each pair was detached and allowed to ripen at 22°C, the other was ripened on the plant. Folates were determined 7 days later, at which time detached and attached fruit had reached the red-ripe stage.

pABAGlu pool sizes

Folate breakdown *in vivo* will produce *p*ABAGlu and *p*ABAGlu$_n$, but efficient salvage is expected to keep their pools small compared with the total folate pool. To test this prediction, total *p*ABAGlu (*p*ABAGlu plus *p*ABAGlu$_n$) levels were assayed by a novel method that entails measuring the *p*ABA released by carboxypeptidase G treatment. This enzyme removes polyglutamyl tails via exopeptidase action and then cleaves glutamate from *p*ABAGlu (McCullough *et al.*, 1971). The liberated *p*ABA is partitioned into ethyl acetate and quantified by fluorometric HPLC.

Total *p*ABAGlu pools in Arabidopsis, pea and tomato tissues ranged from 0.06 to 0.6 nmol g^{-1} FW, which is only 4–14% of typical values for folates in these tissues (Figures 2a and 3a). Moreover, these *p*ABAGlu data overestimate *in vivo* levels because they are inflated by folate breakdown during the assay. Although assay conditions were designed to minimize oxidative folate cleavage, some is inevitable, and tests with folates added to extracts showed that 5-methyl-, 5,10-methenyl-, and 5-formyl-THF underwent up to 4–6% breakdown, and THF up to 26%. The small *p*ABAGlu pools observed can thus be ascribed partly to folate breakdown.

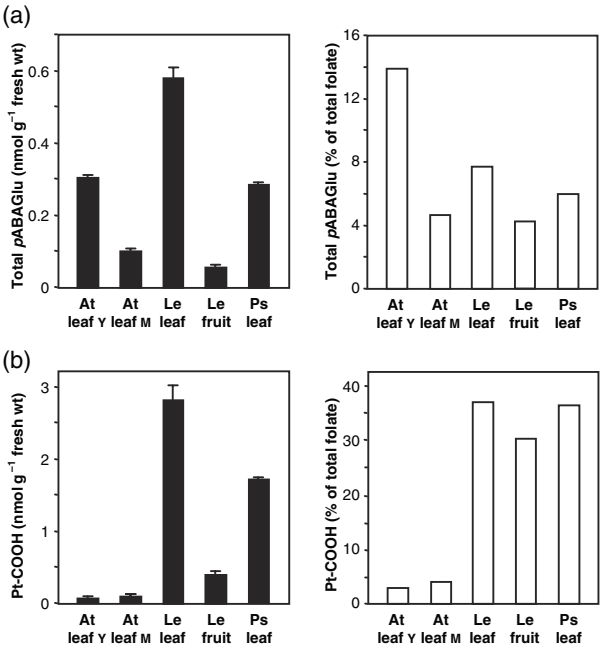

Figure 3. *p*ABAGlu and pterin-6-carboxylate levels in plant tissues.
*p*ABAGlu and pterin-6-carboxylate were determined in Arabidopsis (At), pea (Ps) and tomato (Le) leaves, and in mature green tomato pericarp. Arabidopsis leaves were from rosettes at 3 (Y) or 6 (M) weeks of age. Pea and tomato leaves were from plants 10 days and 4 weeks old, respectively. Data are expressed as g^{-1} FW (means of three replicates and SE) and as a percentage of the measured or reported folate content. Folate contents (nmol g^{-1} FW) used in this calculation were: tomato pericarp, 1.3 (this study); Arabidopsis leaf, 2.2 (Goyer *et al.*, 2005); pea leaf, 4.7 (Chan and Cossins, 2003); tomato leaf, 7.6 (Cossins, 2000). The values for pea and for 3- and 6-week-old Arabidopsis were confirmed by this study. (a) Total *p*ABAGlu (*p*ABAGlu plus *p*ABAGlu$_n$) levels; (b) pterin-6-carboxylate levels.

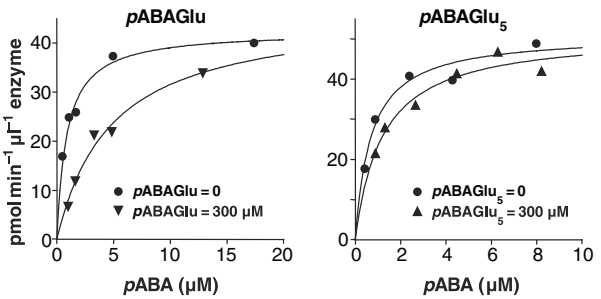

Figure 4. Inhibition of dihydropteroate synthase activity by *p*ABAGlu or *p*ABAGlu$_5$.
Partially purified dihydropteroate synthase (DHPS) from pea mitochondria was assayed radiometrically with various concentrations of [^{14}C]*p*ABA without and with 300 µM *p*ABAGlu or *p*ABAGlu$_5$. The pentaglutamate form was selected for these tests because it is the breakdown product of pentaglutamyl folates, which often dominate the folate pool in plants (Cossins, 2000; Goyer *et al.*, 2005; Orsomando *et al.*, 2005).

Further evidence for salvage of *p*ABAGlu comes from the Arabidopsis experiment in which net folate breakdown occurred due to blockade of folate synthesis with sulfanilamide (an inhibitor of DHPS) (Figure 2a). The net folate loss was accompanied by little accumulation of *p*ABAGlu, but by a marked rise in *p*ABA and its metabolite *p*ABA glucose ester, suggesting that *p*ABAGlu moieties released by folate breakdown are efficiently hydrolysed to *p*ABA (Figure 2a). Note that *p*ABAGlu hydrolysis was not the only source of the rise in *p*ABA, as the build-up of free *p*ABA and its glucose ester (2.48 and 3.21 nmol g^{-1} FW, respectively) far exceeded the net loss of folate (0.41 nmol g^{-1} FW). The extra *p*ABA presumably came from *de novo* synthesis, perhaps accelerated by loss of feedback inhibition of *p*ABA production as folate levels fell.

pABAGlu and dihydropteroate synthase

While the above evidence is consistent with salvage of *p*ABAGlu and *p*ABAGlu$_n$ via conversion to *p*ABA, it does not exclude the possibility of salvage by direct incorporation into DHF via the action of DHPS. To investigate this alternative, we first tested *p*ABAGlu and *p*ABAGlu$_5$ as inhibitors of the well characterized enzyme from pea (Rébeillé *et al.*, 1997). Activity was assayed radiometrically using [^{14}C]*p*ABA as substrate. *p*ABAGlu was a moderate inhibitor of [^{14}C]dihydropteroate synthesis, and *p*ABAGlu$_5$ a weak one (Figure 4). Double-reciprocal plots showed that the inhibition was competitive, and gave K_i values of 30–50 µM for *p*ABAGlu and 400–600 µM for *p*ABAGlu$_5$ (and a K_m value for *p*ABA of approximately 0.8 µM, in accordance with published data). The difference between the K_i values and the K_m for *p*ABA suggests that *p*ABAGlu is – at best – a poor substrate for DHPS, and that *p*ABAGlu$_5$ is an extremely poor one. Semiquantitative radioassays using [^{3}H]*p*ABAGlu as substrate supported the former inference; formation of [^{3}H]DHF was confirmed by HPLC analysis, and the K_m for *p*ABAGlu was estimated as approximately 100 µM (not shown). The large amount of [^{3}H]*p*ABAGlu substrate needed in each assay precluded more detailed kinetic studies.

As physiological context for these kinetic data, note that pea leaves contain ≤0.3 nmol *p*ABAGlu moieties g^{-1} FW (Figure 3a), and that mitochondrial volume is approximately 5 µl g^{-1} FW (Winter *et al.*, 1994). Thus, even in the unlikely scenario where all the leaf's *p*ABAGlu moieties (mono- and polyglutamyl) are assumed to be in mitochondria, their concentration in the matrix would be ≤60 µM.

Hydrolysis of pABAGlu in vivo

Given the above indirect evidence for salvage of *p*ABAGlu by hydrolysis, we tested for this capacity directly. *p*ABAGlu labelled with ^{14}C in the *p*ABA moiety was supplied to Arabidopsis leaf or tomato pericarp tissue, and after 6 h the absorbed and non-absorbed ^{14}C was analysed. Both tissues took up 80–90% of the [^{14}C]*p*ABAGlu and hydrolysed significant proportions: 5.4% of the absorbed label in leaf tissue; 11% in pericarp (Figure 5a). Hydrolysis was assessed by summing the ^{14}C present in free *p*ABA, its glucose ester (a major metabolite of free *p*ABA; Quinlivan *et al.*, 2003) and its *N*-glucoside (a *p*ABA adduct formed during extraction; Quinlivan *et al.*, 2003) (Figure 5b). The hydrolysis probably occurred intracellularly, as the non-absorbed label was almost all in the form of *p*ABAGlu (Figure 5a). The amounts of hydrolysis in leaves and pericarp were equivalent to rates of 1.5 and 2.6 nmol g^{-1} FW day^{-1}, respectively (assuming rates are constant). These estimated rates are high relative to typical total folate pools (2.2 and 1.3 nmol g^{-1} FW in leaves and pericarp, respectively; Figure 3).

Pterin-6-carboxylate pool sizes

As with *p*ABAGlu, folate breakdown will produce pterin products, but if salvage reactions exist they should not accumulate. Pterin-6-carboxylate is expected to be diagnostic in this respect as it is a stable end-product formed by oxidation of the first products of folate cleavage (Figure 1a); it is not salvaged by bacteria (Shiota, 1959) or *Leishmania* (Nare *et al.*, 1997), and probably not by plants (Stakhov *et al.*, 2002). A lack of pterin-6-carboxylate accumulation would thus suggest pterin salvage activity. Pterin-6-carboxylate levels were relatively low, from 0.06 to 2.8 nmol g^{-1} FW, and were equivalent to 3–37% of typical folate levels (Figure 3b).

Reduction of dihydropterin-6-aldehyde and pterin-6-aldehyde in vivo

Dihydropterin-6-aldehyde is a primary folate-oxidation product that potentially can be salvaged by reduction to hydroxymethyldihydropterin (Figure 1a). This reaction was

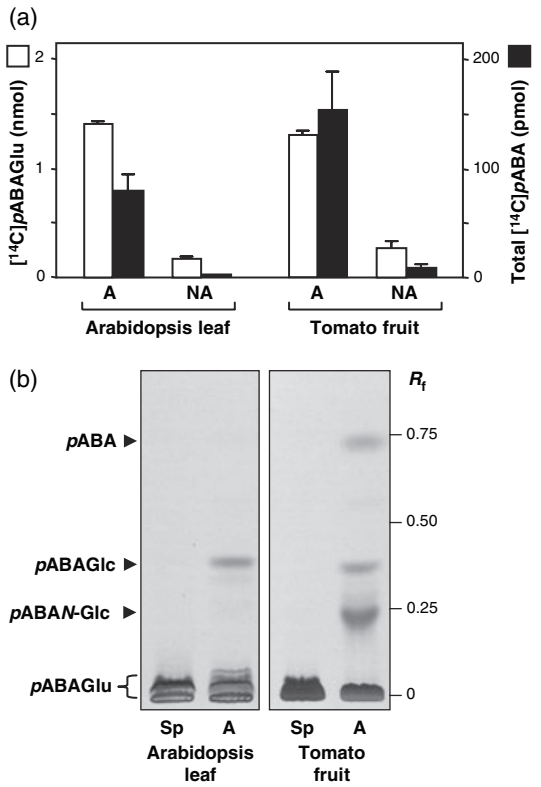

Figure 5. *In vivo* hydrolysis of [^{14}C]*p*ABAGlu.
Arabidopsis leaf and mature green tomato pericarp tissues (mean FW 0.20 and 0.23 g, respectively) were given 1.7 nmol (92 nCi) of [^{14}C]*p*ABAGlu for 6 h in the light. Absorbed and non-absorbed radiolabel was then analysed by TLC to determine how much remained as *p*ABAGlu and how much was in the form of *p*ABA or its glucose ester (a *p*ABA metabolite) and *N*-glucoside (formed from *p*ABA during extraction). Arabidopsis leaves were from rosettes of 6-week-old plants.
(a) Quantification of radiolabelled *p*ABAGlu and total *p*ABA (= free *p*ABA plus its glucose ester and *N*-glucoside) in absorbed (A) and non-absorbed (NA) fractions. *p*ABAGlu data are expressed in nmol; total *p*ABA data in pmol. Data are means of three or four replicates and SE, and are corrected for recovery.
(b) Autoradiograph of a TLC separation of representative Arabidopsis leaf and tomato pericarp absorbed fractions (A), along with fractions spiked with 28 nCi of [^{14}C]*p*ABAGlu before extraction (Sp) for comparison. The running positions of *p*ABAGlu, *p*ABA glucose ester (*p*ABAGlc) and *p*ABA *N*-glucoside (*p*ABA *N*-Glc) are indicated, as are R_f values.

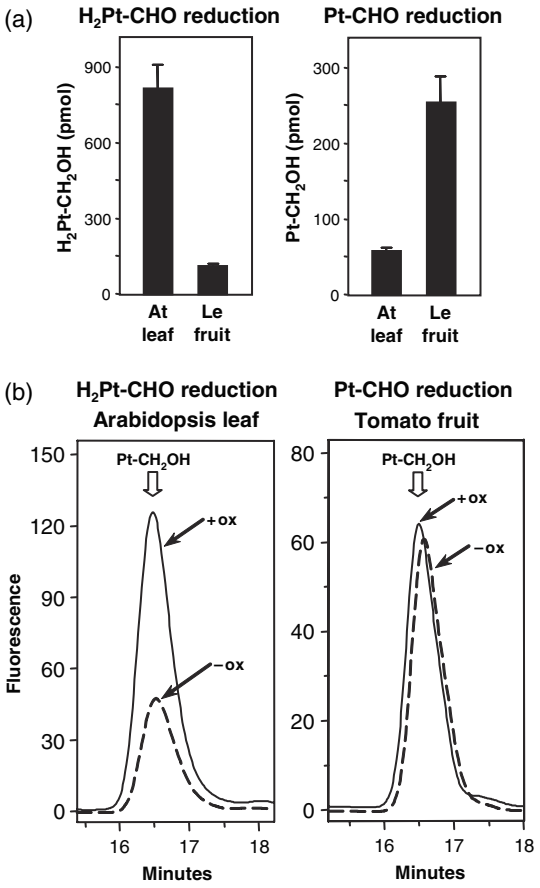

Figure 6. *In vivo* reduction of dihydropterin-6-aldehyde and pterin-6-aldehyde to the corresponding hydroxymethyl compounds.
Arabidopsis leaf and mature green tomato pericarp tissues (mean FW 0.10 and 0.20 g, respectively) were given 3.4 nmol of dihydropterin-6-aldehyde (H_2Pt-CHO) or 4.9 nmol of pterin-6-aldehyde (Pt-CHO) for 14.5 h in darkness, and washed for 0.5 h. Formation of hydroxymethyldihydropterin (H_2Pt-CH_2OH) or hydroxymethylpterin (Pt-CH_2OH) was then measured; the amounts of both compounds in controls given no pterin were negligible. Hydroxymethyldihydropterin was estimated from the increase in the hydroxymethylpterin peak after oxidation.
(a) Dihydropterin-6-aldehyde reduction to hydroxymethyldihydropterin, and pterin-6-aldehyde reduction to hydroxymethylpterin. Data are means of three replicates and SE, and are corrected for recovery.
(b) The hydroxymethylpterin region of HPLC profiles of representative samples, showing the peak in tissues fed dihydropterin-6-aldehyde (left panel) or pterin-6-aldehyde (right panel). Samples were analysed without (−ox) and with (+ox) oxidation before HPLC. Note the large increase in the peak on oxidation of the dihydropterin-6-aldehyde-fed sample, and the lack of such an increase on oxidation of the pterin-6-aldehyde-fed sample.

investigated *in vivo* by measuring the accumulation of hydroxymethyldihydropterin in tissues fed with dihydropterin-6-aldehyde for 15 h. Hydroxymethyldihydropterin was estimated by fluorometric HPLC from the difference in hydroxymethylpterin peak size between oxidized and non-oxidized portions of each sample. Arabidopsis leaf and tomato pericarp tissues readily converted dihydropterin-6-aldehyde to hydroxymethyldihydropterin, at rates equivalent to 12 and 0.9 nmol g^{-1} FW day^{-1}, respectively (Figure 6). These rates are similar to, or above, those for *p*ABAGlu hydrolysis.

We also tested whether the later folate-oxidation product, pterin-6-aldehyde, could be salvaged by reducing the aldehyde side chain and the pterin ring (Figure 6). Side-chain reduction (giving hydroxymethylpterin) was readily detectable in leaf and pericarp tissue, but not side-chain-plus-ring reduction (giving hydroxymethyldihydropterin). It is noteworthy that pericarp was more active than leaf tissue in pterin-6-aldehyde reduction, while the reverse was true of dihydropterin-6-aldehyde reduction: in the simplest case, this suggests that more than one enzyme is involved.

Table 1 Folate salvage enzyme activities in desalted protein extracts

Tissue	*p*ABAGlu hydrolysis (nmol h^{-1} mg^{-1} protein)	NADPH-dependent aldehyde group reduction (nmol h^{-1} mg^{-1} protein)	
		Dihydropterin-6-aldehyde	Pterin-6-aldehyde
Pea leaf	0.026 ± 0.002	21.5 ± 0.5	45.7 ± 0.5
Arabidopsis leaf	0.137 ± 0.010	1.79 ± 0.03	4.31 ± 0.06
Tomato pericarp	1.86 ± 0.09	0.45 ± 0.05	26.0 ± 0.2

Values are means of three replicates ±SE. Initial substrate concentrations were: *p*ABAGlu, 83 μM; dihydropterin-6-aldehyde and pterin-6-aldehyde, 10 μM; NADPH, 1 mM. Pea and Arabidopsis leaves were 10 days and 4 weeks old, respectively. Tomato pericarp was at the mature green stage.

Demonstration of salvage reactions in vitro

To corroborate the *in vivo* evidence for *p*ABAGlu hydrolysis and dihydropterin-6-aldehyde reduction, the same reactions were sought *in vitro* using desalted protein extracts of Arabidopsis and pea leaves, and tomato pericarp (Table 1). Both reactions were easily detected, with NADPH as cofactor for the reduction. Their relative activities varied, with *p*ABAGlu hydrolysing activity in the order tomato > Arabidopsis > pea; and dihydropterin-6-aldehyde reduction in the opposite order. Even the lowest of these activities, *p*ABAGlu hydrolysis in pea leaves (0.026 nmol h^{-1} mg^{-1} protein), was quite high relative to the folate content of the source tissue (approximately 0.2 nmol mg^{-1} protein; Chan and Cossins, 2003). As with the *in vivo* results, the reduction of pterin-6-aldehyde to hydroxymethylpterin was also detected, but not the further reduction to hydroxymethyldihydropterin (Table 1). Also as with *in vivo* data, tomato pericarp was more active than Arabidopsis with pterin-6-aldehyde as substrate, and less active with dihydropterin-6-aldehyde.

Discussion

Strikingly little is known about folate salvage in any organism, given the lability of folates and the plentiful data on salvage of more stable cofactors such as nucleotides, NAD^+, and pyridoxal 5′-phosphate (Neidhardt, 1996). In the case of plants, this lack of information is unfortunate as fruits and vegetables – major folate sources for humans – are subject to large post-harvest folate losses, and declining salvage capacity could be among the causes. Moreover, leaves endure oxidative and UVB stresses (Fryer *et al.*, 2002; Gorton and Vogelmann, 1996) that destroy folates, pointing to the existence of robust adaptive mechanisms, of which salvage may well be one.

We first designed a method to measure total *p*ABAGlu, as the standard procedure (McPartlin *et al.*, 1992) does not detect *p*ABAGlu$_n$ and is set up for urine, which has far fewer potential interferants than plant tissues. Our method – built on the specificity of carboxypeptidase G and on the partitioning properties of *p*ABA – worked well on diverse samples and provided the first estimates of *p*ABAGlu pool sizes in plants. To help optimize this method, and to assay *p*ABAGlu hydrolysis, we also developed an efficient radiochemical synthesis for *p*ABAGlu from [^{14}C]*p*ABA. This procedure was shown also to work well for [^{3}H]*p*ABAGlu, thus providing an economical alternative to the usual protocol based on cleaving [^{3}H]folic acid (McPartlin *et al.*, 1992).

The data we obtained strengthen the case for substantial folate-breakdown rates in plant tissues, and indicate that the breakdown products can be salvaged for re-use. While none of the individual strands of evidence is unequivocal, they are collectively quite compelling, as discussed below.

Respecting breakdown, blocking folate synthesis with sulfanilamide and monitoring folate disappearance suggested that the rate in Arabidopsis leaves under such conditions is approximately 10% per day (assuming it to be constant), and that it is normally countered by *de novo* or salvage synthesis. A similar rate of net breakdown was seen in detached tomato fruit; the true breakdown rate may have been higher as some synthesis could have occurred. Note, however, that sulfanilamide treatment disrupts C_1 metabolism (Prabhu *et al.*, 1998), and that this may affect folate-breakdown rates. Likewise, care is needed in extrapolating post-harvest folate-breakdown rates to intact plants as harvest may affect both synthesis and breakdown processes.

The evidence for folate salvage reactions is fourfold. First, *p*ABAGlu moieties did not accumulate greatly in any tissue tested, the levels being certainly no more than 4–14% that of folate, and probably less. Supposing folate-breakdown rates to be approximately 10% per day, such *p*ABAGlu pools would represent, at most, one day's folate breakdown, from which it can be inferred that *p*ABAGlu is salvaged or otherwise metabolized. Similarly, the characteristic pterin end product pterin-6-carboxylate did not accumulate massively, although its levels in three tissues surpassed those of *p*ABAGlu. Although this difference might suggest that pterins are less efficiently salvaged than *p*ABAGlu, it is possible that folate breakdown was not the sole source of pterin-6-carboxylate, as this can, in principle, also come from intermediates of folate synthesis (Rembold, 1985).

A second line of evidence for salvage is that *p*ABAGlu pools in Arabidopsis changed little during sulfanilamide

treatment despite a large concurrent folate loss, whereas the free *p*ABA pool expanded. These observations confirm the above inference that *p*ABAGlu is readily metabolized, and are consistent with the view that its main fate is hydrolysis to *p*ABA and Glu.

The last – and most direct – lines of evidence for salvage reactions are that plant tissues readily converted supplied folate breakdown products to folate synthesis precursors *in vivo* and that protein extracts did so *in vitro*. *p*ABAGlu was hydrolysed to give *p*-aminobenzoate, and dihydropterin-6-aldehyde was reduced to hydroxymethyldihydropterin; the oxidized form of the latter, pterin-6-aldehyde, was likewise reduced to hydroxymethylpterin.

Although our observations do not directly prove that folate-breakdown products are salvaged *in vivo*, it may be strongly inferred that this occurs for *p*ABAGlu at least. Specifically, there is abundant evidence that plants convert free *p*ABA to folates (Imeson *et al.*, 1990; Zheng *et al.*, 1992) and none that they catabolize it. The demonstration that plants release *p*ABA from *p*ABAGlu is thus essentially tantamount to showing that *p*ABAGlu is salvaged.

Neither *in vivo* nor *in vitro* tests with pterin-6-aldehyde as substrate demonstrated reduction of the pterin ring to the dihydro form, a salvage reaction well known in *Leishmania* (Nare *et al.*, 1997). Nor did parallel trials with hydroxymethylpterin show measurable ring reduction (A.D.H. and E.P.Q., unpublished data). It would, however, be premature to conclude that plants lack such a capacity. For example, if the ring-reduction reaction was specific for pterin-6-aldehyde, and much slower than reduction of the aldehyde side chain, our experiments would not have detected it.

Salvage of *p*ABAGlu moieties by direct reincorporation into dihydrofolate via the action of DHPS seems unlikely from the properties of this enzyme. Like its microbial counterparts, plant DHPS can use *p*ABAGlu, but apparently too inefficiently for this reaction to be significant *in vivo* given the low levels of *p*ABAGlu moieties in plant tissues. Furthermore, in effect DHPS can use only *p*ABAGlu, not its polyglutamylated forms, but this enzyme is located in mitochondria (Rébeillé *et al.*, 1997) whereas the only enzyme known to remove polyglutamyl tails, γ-glutamyl hydrolase, is vacuolar (Orsomando *et al.*, 2005). This arrangement imposes a lengthy trajectory with various transport steps on *p*ABAGlu$_n$ before it could be recycled by DHPS.

In summary, this work demonstrates that folate breakdown can occur at relatively high rates in plants, and provides unequivocal evidence for reactions that salvage the breakdown products. It further shows that (i) *p*ABAGlu and its polyglutamyl forms are most probably recycled via prior conversion to free *p*ABA, thereby pointing to a central role for *p*ABAGlu hydrolase activity in the salvage process; and (ii) a primary pterin product of folate breakdown, dihydropterin-6-aldehyde, can potentially be recycled by reducing the 6-aldehyde group, thereby implicating a pterin aldehyde reductase. More generally, our data indicate that plants are excellent models to investigate folate salvage, an area of biochemistry that has been almost completely neglected in plants, fungi and bacteria.

Experimental procedures

Materials

Pterins, *p*ABAGlu and *p*ABAGlu$_5$ were from Schircks Laboratories (Jona, Switzerland). 6-Hydroxymethylpterin pyrophosphate was reduced to its dihydro form as detailed (Scrimgeour, 1980). *p*ABA-glucose ester and -*N*-glucoside were prepared as described (Quinlivan *et al.*, 2003). [*Ring*-^{14}C]*p*ABA (55 mCi mmol^{-1}) was from Moravek Biochemicals (Brea, CA, USA). Dowex resins were from Bio-Rad (Hercules, CA, USA). TLC plates were from Merck (Darmstadt, Germany).

Radiosynthesis of pABAGlu

Reactions were protected from light. Drying steps were *in vacuo* without heating. [^{14}C]*p*ABA (10 μCi, 180 nmol) was dried in a 1.5-ml tube and treated for 2 h at 22°C with 50 μl acetic anhydride with occasional mixing. The product was dried, retreated with acetic anhydride, and redried. The *N*-acetyl-*p*ABA product was then mixed with 8 μl 1 M $NaHCO_3$ (brought to pH 4 with HCl), 8 μl 1 M L-glutamate diethyl ester, and 20 μl 1 M *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride. After 1 h at 22°C, the reaction mixture was applied to a 2-cm origin on a 10-cm silica gel 60 F_{254} TLC plate, which was developed with ethyl acetate:methanol:water (77:13:10, v/v/v). The *N*-acetyl-*p*ABAGlu diethyl ester zone ($R_f \approx 0.6$) was located by quenching of the fluorescent indicator or by autoradiography, scraped from the plate, and eluted with 3 × 200 μl 80% methanol. The eluate was dried, dissolved in 60 μl 0.2 M HCl, transferred to a 1-ml glass vial closed with a Teflon-faced septum, and heated for 45 min at 100°C. The reaction products were dried, redissolved in 20 μl water, and applied to a 3-cm origin on a TLC plate as above, which was developed with ethyl acetate:methanol:water (57:23:20, v/v/v). The *p*ABAGlu zone ($R_f \approx 0.4$) was located and eluted as above. The eluate was dried and redissolved in 200 μl water. Radiochemical yield was approximately 20% and radiochemical purity was >98%.

Plants and growing conditions

Arabidopsis plants (ecotype Columbia) were grown in potting soil in chambers (8-h days at 18–22°C or 12-h days at 23–28°C, 80–90 μmol photons m^{-2} sec^{-1}). Plants for *in vivo* pterin-metabolism experiments were grown in 8-h days for 7–8 weeks; other plants were grown in 12-h days for times given in the text. Arabidopsis plantlets were cultured axenically in 0.5× liquid MS salts containing 10 g l^{-1} sucrose (Prabhu *et al.*, 1996). Tomato plants (cv. Micro-Tom) were grown as described in a chamber (Díaz de la Garza *et al.*, 2004) or glasshouse (Simkin *et al.*, 2004). Pea plants (cv. Laxton's Progress 9) were grown in a chamber as described (Orsomando *et al.*, 2005).

pABAGlu analysis

The procedure entailed: (i) extraction conditions designed to minimize folate breakdown; (ii) an initial step to deplete free *p*ABA by partitioning it at pH ≈ 5.5 into ethyl acetate; (iii) digestion of the

*p*ABA-depleted extract at pH ≈ 7.2 by *Pseudomonas* carboxypeptidase G (Sigma, St. Louis, MO, USA), which removes γ-glutamyl tails from *p*ABAGlu$_n$ and cleaves *p*ABAGlu to *p*ABA plus Glu (McCullough *et al.*, 1971); (iv) partitioning of the *p*ABA so released into ethyl acetate at pH ≈ 5.5, followed by back-extraction; and (v) quantification of *p*ABA by HPLC. Tissues were pulverized in liquid N_2, and 100-mg portions of the frozen powder were extracted for 5 min at 100°C in 0.8 ml N_2-sparged 50 mM 2-(N-morpholino)-ethanesulfonic acid (MES)–KOH containing 0.1% (w/v) sodium ascorbate and 10 mM β-mercaptoethanol, and adjusted to pH 5.5. After cooling and centrifuging, a 0.75-ml aliquot of the extract was readjusted if necessary to pH 5.3–5.8 with 1 M HCl, then shaken for 5 min with 4 ml ethyl acetate that had been pre-equilibrated with 50 mM MES–KOH pH 5.2. The ethyl acetate phase was discarded. The aqueous phase was adjusted to pH 7.0–7.3 with 1 M KOH, mixed with 7.5 μl 10 mM $ZnCl_2$ and 0.1 U carboxypeptidase G (in 10 μl of 50 mM Tris–HCl pH 7.0), and incubated at 37°C for 4–5 h. The pH was then readjusted to 5.3–5.8, followed by partitioning against 4 ml ethyl acetate, as above. The ethyl acetate phase was back-extracted by shaking for 5 min with 0.2 ml 0.3 M KOH. The KOH phase was withdrawn, its volume measured, and 50-μl samples were injected onto a Supelco (St. Louis, MO, USA) Discovery C_{18} column (4.6 × 250 mm, 5 μm particle size) and quantified by fluorescence (270 nm excitation, 350 nm emission). The column was eluted at 1 ml min^{-1} with a 6-min linear gradient from 2 to 29% methanol in 50 mM sodium phosphate buffer pH 6.0, containing 8 mM tetrabutylammonium bisulfate. Each batch of assays included a blank without carboxypeptidase, and samples spiked with 50 pmol *p*ABAGlu or *p*ABAGlu$_5$ from which recovery values were calculated and used to correct the data. Recoveries of *p*ABAGlu and *p*ABAGlu$_5$ were typically 40–60%. Representative samples were spiked with 200–250 pmol THF or its 5-methyl, 10-formyl or 5-formyl derivatives to assess the amount of *p*ABAGlu formed by folate breakdown during the assay.

Pterin analysis

For analysis of endogenous pterin-6-carboxylate, tissue (0.2 g FW) was ground in liquid N_2, suspended in 3.6 ml methanol:chloroform:water (12:5:1, v/v/v), heated to 80°C for 5 min, and vortex mixed for 5 min. After clearing by centrifugation, extracts were split into aqueous and organic phases by adding 0.5 ml chloroform and 0.75 ml water, mixed thoroughly, and centrifuged. The aqueous phase was dried *in vacuo* without heating, taken up in 2 ml water, and fractionated by an ion-exchange procedure based on that of Stea *et al.* (1980). The 2-ml sample was applied to a 0.6-ml, 7-mm diameter column of AG-50WX8 (H^+), and the flow-through and a 15-ml water wash were run straight onto a 0.4-ml, 7-mm diameter column of AG1-X8 (OH^-). The AG1-X8 column was then washed with 6 ml water and eluted with 2 ml 1 M HCl containing 14% (v/v) methanol and 10% (v/v) acetonitrile. The eluates were dried as above and redissolved in 0.2 ml water. No reduced pterins remained in plant samples prepared as above, so no oxidation step was needed to convert pterins to their fully oxidized, fluorescent forms before HPLC. Recoveries of pterin-6-carboxylate spikes were 63–86%; these values were used for data correction.

For pterin metabolism experiments, tissues were ground as above and extracted at 50°C for 5 min in 2 ml methanol:chloroform:water containing 0.1% (w/v) sodium ascorbate and 10 mM β-mercaptoethanol. After phase-splitting as above, the aqueous phase was concentrated to approximately 0.05–0.1 ml *in vacuo* without heat, adjusted to 0.2 ml with water, and analysed at once or held under N_2 at −80°C. Just before HPLC analysis, 50-μl aliquots were treated for 1 h with 80 μl 1 N HCl containing 1% I_2/2% KI (w/v) (which oxidizes reduced pterins) or with 1 N HCl alone (which does not oxidize pterins). All samples then received 5 μl 5% (w/v) sodium ascorbate (to destroy excess I_2) and 7 μl 10 N NaOH. Recoveries of 1-nmol spikes of hydroxymethylpterin and its dihydro form were 65 and 31–46%, respectively; these values were used for data correction.

Pterins (40- or 50-μl injections) were separated by HPLC using a 4-μm, 250 × 4.6-mm Synergi Fusion-RP 80 column (Phenomenex, Torrance, CA, USA) eluted isocratically with 10 mM sodium phosphate (pH 6.0) at 1.5 ml min^{-1}. Peaks were detected by fluorescence (350 nm excitation, 450 nm emission) and quantified relative to standards. Hydroxymethyldihydropterin was estimated from the increase in the hydroxymethylpterin peak on oxidation. The identity of the hydroxymethylpterin peak was confirmed by LC–MS from the presence of the molecular ion ($M + H^+$) at *m/z* 194, and diagnostic fragment ions at *m/z* 176, 134 and 106. Pterin concentrations in standard solutions were determined using published extinction coefficients (Blakley, 1969; Pfleiderer, 1985), except for dihydropterin-6-aldehyde, for which we estimated a value of 10 400 M^{-1} cm^{-1} at 422 nm and pH 7.5. Solutions of dihydropterins contained 10 mM β-mercaptoethanol and were kept under N_2.

Analysis of folates, pABA and pABA glucose ester

Folates were extracted from samples (0.5–1 g FW), deglutamylated, affinity purified, and determined by HPLC with electrochemical detection as described (Díaz de la Garza *et al.*, 2004; Goyer *et al.*, 2005). Free and total *p*ABA were determined as described (Quinlivan *et al.*, 2003), except that the ion-exchange step was omitted. Instead, a final concentration of 50 mM MES–KOH (pH 5.2) was added to each sample (both unhydrolysed and acid-hydrolysed) and the pH was adjusted to 4.6–5.0 with NaOH. The *p*ABA was then extracted into ethyl acetate as before. Using this method, <0.1% *p*ABA glucose ester was converted to *p*ABA in the absence of acid hydrolysis. The difference between free and total *p*ABA values was taken to be *p*ABA glucose ester.

Metabolism of [^{14}C]pABAGlu in vivo

Samples consisted of (i) five Arabidopsis rosette leaves trimmed to 2 cm long and stripped of the mid-vein on the abaxial surface; or (ii) single 1-cm-diameter pericarp disks from mature green tomato fruit, given eight radial cuts on the inner surface. [^{14}C]*p*ABAGlu (92 nCi, 1.7 nmol) in 10 μl water was supplied to the cut surfaces (2 μl per leaf or 10 μl per disk) followed, after uptake, by 2 μl water in the case of leaf sections. Samples were then incubated on moist filter paper in Petri dishes at 26°C for 6 h in fluorescent light (150 μmol photons m^{-2} sec^{-1}). Non-absorbed ^{14}C was washed out by shaking samples for 15 min in 5 ml 20 μM unlabelled *p*ABAGlu. An aliquot (1.5 ml) of the washing out solution was dried *in vacuo* for TLC analysis (see below). Tissues were extracted by grinding in 2 ml semi-frozen methanol and warming to 60°C for 5 min, then re-extracted by shaking in 0.5 ml water at 22°C for 5 min. The methanol and water extracts were pooled, and 0.2-ml aliquots were dried *in vacuo*, redissolved in 5 μl water, mixed with unlabelled standards (*p*ABA, *p*ABA glucose ester, and *p*ABA *N*-glucoside), and applied to 1-cm origins on TLC plates as above. Plates were developed with ethyl acetate:methanol:water (77:13:10, v/v/v) and autoradiographed to locate labelled zones, which were scraped out for ^{14}C assay by scintillation counting. Unlabelled samples spiked with [^{14}C]*p*ABAGlu were used to correct for slight (approximately 0.5%) chemical decomposition of [^{14}C]*p*ABAGlu during extraction, and for recovery.

Metabolism of pterins in vivo

Tissue samples were as for [^{14}C]*p*ABAGlu experiments. Samples received 20 μl 10 mM potassium phosphate pH 7.5, alone (control) or containing 3.4 nmol dihydropterin-6-aldehyde or 4.9 nmol pterin-6-aldehyde; the dihydropterin solution also contained 10 mM β-mercaptoethanol. After incubating on moist filter paper in darkness for 14.5 h at 22°C, non-absorbed pterins were washed out by shaking in 5 ml water for 30 min. Extracts were prepared and analysed for hydroxymethyldihydropterin and hydroxymethylpterin by HPLC, as above. Spikes of dihydropterin-6-aldehyde and pterin-6-aldehyde confirmed that unmetabolized aldehydes did not give rise to significant amounts of the corresponding hydroxymethyl compounds during sample work-up.

Tissue extracts and enzyme assays

Tissues were ground in liquid N_2. Subsequent operations were at 0–10°C. For *p*ABAGlu hydrolysis assays, the frozen powder was extracted in 3.5 vol 50 mM potassium phosphate pH 8, containing 3% (w/v) polyvinylpolypyrolidone. The brei was centrifuged (10 000 ***g***, 20 min) and the supernatant was brought to 80% saturation with $(NH_4)_2SO_4$. The precipitated proteins were resuspended in 50 mM potassium phosphate pH 8, desalted on a PD10 column equilibrated with the same buffer plus 10% (v/v) glycerol, flash-frozen, and stored at −80°C. Protein was estimated by dye-binding (Bradford, 1976). *p*ABAGlu hydrolysis was assayed in 10-μl reactions containing 8 μl protein extract (8–100 μg protein), 10 nmol $MnCl_2$, and 46 nCi (0.83 nmol) of [^{14}C]*p*ABAGlu. After incubating for 3 h at 30°C, 0.1 ml 0.1 M sodium citrate buffer pH 5.5 was added and the mixture was shaken for 5 min with 0.6 ml ethyl acetate. The [^{14}C]*p*ABA partitioned into the organic phase was quantified by scintillation counting. Substrate consumption in the assays was <10%.

For pterin-reduction assays, tissues were ground as above and extracted with 2 vol 100 mM potassium phosphate pH 7.5 containing 5 mM dithiothreitol and 3% (w/v) polyvinylpolypyrolidone. After centrifuging, the supernatant was desalted on a PD10 column equilibrated with 50 mM potassium phosphate pH 7.5 plus 5 mM dithiothreitol and 10% glycerol, and frozen as above. Assays (final volume 50 μl, pH 7.5) contained 4–20-μl protein extract (17–29 μg protein), 1.8 μmol potassium phosphate, 0.5 nmol pterin and 50 nmol NADPH; blanks contained no NADPH. After 15–30 min incubation at 30°C, 20-μl samples were treated for 1 h with 10 μl 1 N HCl containing 1% I_2/2% KI or with 1 N HCl alone, after which they received 10 μl 5% sodium ascorbate and 60 μl 10 mM sodium phosphate pH 6.0, containing 1% (w/v) Na-ascorbate and 10 mM β-mercaptoethanol (to stabilize dihydropterins). Hydroxymethylpterin and its dihydro form were assayed by HPLC as above. Pterin utilization in the assays was ≤50% and NADPH consumption was ≤13%.

Dihydropteroate synthase studies

Dihydropteroate synthase activity was partially purified from pea leaf mitochondria as described (Rébeillé *et al.*, 1997). Soluble proteins from purified mitochondria were loaded on a Superdex 200 (Pharmacia, Piscataway, NJ, USA) column (60 × 1 cm) pre-equilibrated with the following buffer: 10 mM potassium phosphate, 10 mM Tris pH 7.2, 1 mM EDTA, 1 mM dithiothreitol, 10 mM β-mercaptoethanol, 10% (w/v) glycerol. Proteins were eluted with the same buffer (flow rate 0.3 ml min^{-1}). Fractions containing DHPS activity were combined and concentrated to 2 ml by ultrafiltration on a 10-kDa cut-off membrane. DHPS activity was assayed in reaction mixtures (final volume 120 μl) containing 40 mM Tris–HCl (pH 8.0), 20 mM β-mercaptoethanol, 10 mM $MgCl_2$, 40 μg partially purified protein and various amounts of *p*ABAGlu or *p*ABAGlu$_5$. Two μl 2 mM [*carboxyl*-^{14}C]*p*ABA (50 mCi mmol^{-1}) were added, then the reaction was started by adding 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (final concentration 100 μM). [^{14}C]7,8-Dihydropteroate formation was estimated by reverse-phase HPLC and scintillation counting as described (Mouillon *et al.*, 2002).

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References

Basset, G., Quinlivan, E.P., Ziemak, M.J., Díaz de la Garza, R., Fischer, M., Schiffmann, S., Bacher, A., Gregory, J.F., III and Hanson, A.D. (2002) Folate synthesis in plants: the first step of the pterin branch is mediated by a unique bimodular GTP cyclohydrolase I. *Proc. Natl Acad. Sci. USA*, **99**, 12489–12494.

Bello, A.R., Nare, B., Freedman, D., Hardy, L. and Beverley, S.M. (1994) PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*. *Proc. Natl Acad. Sci. USA*, **91**, 11442–11446.

Blakley, R.L. (ed.) (1969) Chemical and physical properties of pterins and folate derivatives. In *The Biochemistry of Folic Acid and Related Pteridines*. New York: Wiley, pp. 58–105.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Brown, G.M., Weisman, R.A. and Molnar, D.A. (1961) The biosynthesis of folic acid. I. Substrate and cofactor requirements for enzymatic synthesis by cell-free extracts of *Escherichia coli*. *J. Biol. Chem.* **236**, 2534–2543.

Chan, S.Y. and Cossins, E.A. (2003) The intracellular distribution of folate derivatives in pea leaves. *Pteridines*, **14**, 67–76.

Cossins, E.A. (2000) The fascinating world of folate and one-carbon metabolism. *Can. J. Bot.* **78**, 691–708.

Díaz de la Garza, R., Quinlivan, E.P., Klaus, S.M., Basset, G.J., Gregory, J.F., III and Hanson, A.D. (2004) Folate biofortification in tomatoes by engineering the pteridine branch of folate synthesis. *Proc. Natl Acad. Sci. USA*, **101**, 13720–13725.

Ferone, R. (1973) The enzymic synthesis of dihydropteroate and dihydrofolate by *Plasmodium berghei*. *J. Protozool.* **20**, 459–464.

Fryer, M.J., Oxborough, K., Mullineaux, P.M. and Baker, N.R. (2002) Imaging of photo-oxidative stress responses in leaves. *J. Exp. Bot.* **53**, 1249–1254.

Gorton, H.L. and Vogelmann, T.C. (1996) Effects of epidermal cell shape and pigmentation on optical properties of antirrhinum petals at visible and ultraviolet wavelengths. *Plant Physiol.* **112**, 879–888.

Goyer, A., Collakova, E., Díaz de la Garza, R., Quinlivan, E.P., Williamson, J., Gregory, J.F., III, Shachar-Hill, Y. and Hanson, A.D. (2005) 5-Formyltetrahydrofolate is an inhibitory but well tolerated metabolite in *Arabidopsis* leaves. *J. Biol. Chem.* **280**, 26137–26142.

Green, J.C., Nichols, B.P. and Matthews, R.G. (1996) Folate biosynthesis, reduction, and polyglutamylation. In *Escherichia coli* and *Salmonella* – Cellular and Molecular Biology, Vol. 1, 2nd edn

(Neidhardt, F.C., ed.). Washington, D.C., USA: ASM Press, pp. 665–673.

Gregory, J.F., III (1989) Chemical and nutritional aspects of folate research: analytical procedures, methods of folate synthesis, stability, and bioavailability of dietary folates. *Adv. Food Nutr. Res.* **33**, 1–101.

Gregory, J.F., III and Quinlivan, E.P. (2002) *In vivo* kinetics of folate metabolism. *Annu. Rev. Nutr.* **22**, 199–220.

Hillcoat, B.L., Nixon, P.F. and Blakley, R.L. (1967) Effect of substrate decomposition on the spectrophotometric assay of dihydrofolate reductase. *Anal. Biochem.* **21**, 178–189.

Hussein, M.J., Green, J.M. and Nichols, B.P. (1998) Characterization of mutations that allow *p*-aminobenzoyl-glutamate utilization by *Escherichia coli*. *J. Bacteriol.* **180**, 6260–6268.

Imeson, H.C., Zheng, L.L. and Cossins, E.A. (1990) Folylpolyglutamyl derivatives of *Pisum sativum* L. Determination of polyglutamate chain lengths by high performance liquid chromatography following conversion to *p*-aminobenzoylpolyglutamates. *Plant Cell Physiol.* **31**, 223–231.

Lowry, O.H., Bessey, O.A. and Crawford, E.J. (1949) Photolytic and enzymatic transformations of pteroylglutamic acid. *J. Biol. Chem.* **180**, 389–398.

McCullough, J.L., Chabner, B.A. and Bertino, J.R. (1971) Purification and properties of carboxypeptidase G1. *J. Biol. Chem.* **246**, 7207–7213.

McPartlin, J., Courtney, G., McNulty, H., Weir, D. and Scott, J. (1992) The quantitative analysis of endogenous folate catabolites in human urine. *Anal. Biochem.* **206**, 256–261.

Mitsuda, H. and Suzuki, Y. (1971) Enzymatic conversion of 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine to 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine by cell-free extracts of *Escherichia coli* B. *J. Vitaminol.* **17**, 5–9.

Mouillon, J.M., Ravanel, S., Douce, R. and Rébeillé, F. (2002) Folate synthesis in higher-plant mitochondria: coupling between the dihydropterin pyrophosphokinase and the dihydropteroate synthase activities. *Biochem. J.* **363**, 313–319.

Nare, B., Hardy, L.W. and Beverley, S.M. (1997) The roles of pteridine reductase 1 and dihydrofolate reductase-thymidylate synthase in pteridine metabolism in the protozoan parasite *Leishmania major*. *J. Biol. Chem.* **272**, 13883–13891.

Neidhardt, F.C. (1996) *Escherichia coli* and *Salmonella* – Cellular and Molecular Biology, Vol. 1, 2nd edn. Washington, D.C., USA: ASM Press.

Orsomando, G., Díaz de la Garza, R., Green, B.J., Peng, M., Rea, P.A., Ryan, T.J., Gregory, J.F., III and Hanson, A.D. (2005) Plant γ-glutamyl hydrolases and folate polyglutamates: characterization, compartmentation, and co-occurrence in vacuoles. *J. Biol. Chem.* **280**, 28877–28884.

Ortiz, P.J. (1970) Dihydrofolate and dihydropteroate synthesis by partially purified enzymes from wild-type and sulfonamide-resistant *Pneumonococcus*. *Biochemistry*, **9**, 355–361.

Pfleiderer, W. (1985) Chemistry of naturally occurring pteridines. In *Folates and Pterins*, Vol. 2 (Blakley, R.L. and Benkovic, S., eds). New York: Wiley, pp. 43–114.

Prabhu, V., Chatson, K.B., Abrams, G.D. and King, J. (1996) ^{13}C Nuclear magnetic resonance detection of interactions of serine hydroxymethyltransferase with C1-tetrahydrofolate synthase and glycine decarboxylase complex activities in Arabidopsis. *Plant Physiol.* **112**, 207–216.

Prabhu, V., Chatson, K.B., Lui, H., Abrams, G.D. and King, J. (1998) Effects of sulfanilamide and methotrexate on ^{13}C fluxes through the glycine decarboxylase/serine hydroxymethyltransferase enzyme system in Arabidopsis. *Plant Physiol.* **116**, 137–144.

Quinlivan, E.P., Roje, S., Basset, G., Shachar-Hill, Y., Gregory, J.F., III and Hanson, A.D. (2003) The folate precursor *p*-aminobenzoate is reversibly converted to its glucose ester in the plant cytosol. *J. Biol. Chem.* **278**, 20731–20737.

Rébeillé, F., Macherel, D., Mouillon, J.M., Garin, J. and Douce, R. (1997) Folate biosynthesis in higher plants: purification and molecular cloning of a bifunctional 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase/7,8-dihydropteroate synthase localized in mitochondria. *EMBO J.* **16**, 947–957.

Reed, L.S. and Archer, M.C. (1980) Oxidation of tetrahydrofolic acid by air. *J. Agric. Food Chem.* **28**, 801–805.

Rembold, H. (1985) Catabolism of pterins. In *Folates and Pterins*, Vol. 2 (Blakley, R.L. and Benkovic, S.J., eds). New York: Wiley, pp. 155–178.

Scott, J.M. (1984) Catabolism of folates. In *Folates and Pterins*, Vol. 1 (Blakley, R.L. and Benkovic, S.J., eds). New York: Wiley, pp. 307–327.

Scott, J., Rébeillé, F. and Fletcher, J. (2000) Folic acid and folates: the feasibility of nutritional enhancement in plant foods. *J. Sci. Food Agric.* **80**, 795–824.

Scrimgeour, K.G. (1980) Methods for reduction, stabilization, and analyses of folates. *Methods Enzymol.* **66**, 517–523.

Sherwood, R.F., Melton, R.G., Alwan, S.M. and Hughes, P. (1985) Purification and properties of carboxypeptidase G2 from *Pseudomonas* sp. strain RS-16. Use of a novel triazine dye affinity method. *Eur. J. Biochem.* **148**, 447–453.

Shiota, T. (1959) Enzymic synthesis of folic acid-like compounds by cell-free extracts of *Lactobacillus arabinosus*. *Arch. Biochem. Biophys.* **80**, 155–161.

Shiota, T., Baugh, C.M., Jackson, R. and Dillard, R. (1969) The enzymatic synthesis of hydroxylmethyldihydropteridine pyrophosphate and dihydrofolate. *Biochemistry*, **8**, 5022–5028.

Simkin, A.J., Schwartz, S.H., Auldridge, M., Taylor, M.G. and Klee, H.J. (2004) The tomato carotenoid cleavage dioxygenase 1 genes contribute to the formation of the flavor volatiles beta-ionone, pseudoionone, and geranylacetone. *Plant J.* **40**, 882–892.

Stakhov, L.F., Ladygin, V.G. and Stakhova, L.N. (2002) Effect of ultraviolet C irradiation on folate and free amino acid contents in leaves of *Pisum sativum*. *Biofizika*, **47**, 878–885.

Stea, B., Halpern, R.M., Halpern, B.C. and Smith, R.A. (1980) Quantitative determination of pterins in biological fluids by high-performance liquid chromatography. *J. Chromatogr.* **188**, 363–375.

Strålsjö, L.M., Witthöft, C.M., Sjöholm, I.M. and Jägerstad, M.I. (2003) Folate content in strawberries (*Fragaria* × *ananassa*): effects of cultivar, ripeness, year of harvest, storage, and commercial processing. *J. Agric. Food Chem.* **51**, 128–133.

Suh, J.R., Herbig, A.K. and Stover, P.J. (2001) New perspectives on folate catabolism. *Annu. Rev. Nutr.* **21**, 255–282.

Swedberg, G., Castensson, S. and Sköld, O. (1979) Characterization of mutationally altered dihydropteroate synthase and its ability to form a sulfonamide-containing dihydrofolate analog. *J. Bacteriol.* **137**, 129–136.

Whiteley, J.M., Drais, J., Kirchner, J. and Huennekens, F.M. (1968) Synthesis of 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine and its identification as a degradation product of dihydrofolate. *Arch. Biochem. Biophys.* **126**, 955–957.

Winter, H., Robinson, D.G. and Heldt, H.W. (1994) Subcellular volumes and metabolite concentrations in spinach leaves. *Planta*, **193**, 530–535.

Zheng, L.L., Lin, Y., Lin, S. and Cossins, E.A. (1992) The polyglutamate nature of plant folates. *Phytochemistry*, **31**, 2277–2282.

