Review

Folate biofortification in food plants

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Folate deficiency is a global health problem affecting many people in the developing and developed world. Current interventions (industrial food fortification and supplementation by folic acid pills) are effective if they can be used but might not be possible in less developed countries. Recent advances demonstrate that folate biofortification of food crops is now a feasible complementary strategy to fight folate deficiency worldwide. The genes and enzymes of folate synthesis are sufficiently understood to enable metabolic engineering of the pathway, and results from pilot engineering studies in plants (and bacteria) are encouraging. Here, we review the current status of investigations in the field of folate enhancement on the eve of a new era in food fortification.

Folates as vitamins and the need for biofortification

Folate is a generic term for tetrahydrofolate (THF) and its derivatives (Figure 1). Folates are B vitamins, necessary in almost all organisms as cofactors for one-carbon (C_1) transfer reactions, generally referred to as C_1-metabolism. Vitally important aspects of C_1-metabolism are nucleotide biosynthesis, amino acid metabolism and the methylation cycle, which supplies numerous methylation reactions with methyl groups (for reviews see Refs [1–3]). Humans and other animals cannot synthesize folates and, therefore, need them in the diet, with plants usually being the main dietary sources [2]. Folate levels vary among food plants; the cereal staples maize, wheat and, particularly, rice contain extremely low levels (USDA National Nutrient Database for Standard Reference. Release 19; http://www.nal.usda.gov/fnic/foodcomp/search/) (Table 1). Reliance on such staples cannot satisfy recommended dietary allowances (RDA), set at 400 μg of dietary folate equivalents (DFE) day^{-1} for adults National Institutes of Health Office of Dietary Supplements Dietary Supplement Fact Sheet: Folate; http://ods.od.nih.gov/factsheets/folate.asp). Clinical and epidemiological evidence shows that folate intake is suboptimal for most populations in developing countries – as well as for surprisingly large population groups in developed countries [4–6]. Suboptimal folate intake perturbs C_1-metabolism, which contributes to megaloblastic anemia, birth defects [neural tube defects (NTD)], and increased risks for cardiovascular disease and certain cancers (Box 1) [7].

Folate deficiency is, therefore, a global health problem. Although fortification and supplementation (vitamin pills) are effective ways to improve folate status, they remain far from accessible to the poor, rural population in developing countries [8,9]. Hence, there is a compelling case for the development of folate-enriched food plants as a sustainable complement to the existing interventions for fighting folate deficiency [9–11]. Recently, major progress has been achieved not only in our understanding of the regulation of the folate biosynthesis pathway, but also in establishing a proof of concept for folate biofortification by metabolic engineering of crops plants. Here, we discuss these achievements, after providing background on the biochemical processes that affect folate content. Research on bacterial and animal systems is included where relevant.

Folate biosynthesis and transport

The steps in folate synthesis are the same in plants and bacteria, and the pathway enzymes and their genes are all known in both groups [12,13]. In essence, the three parts of the THF molecule – the pteridine, *p*-aminobenzoate (p-ABA) and glutamate moieties (Figure 1) – are produced separately and then joined together. In bacteria, the whole process takes place in the cytosol, but in plants three subcellular compartments are involved: plastids, mitochondria and cytosol [14] (Figure 2). The pteridine moiety is formed from guanosine triphosphate (GTP) in the cytosol and the p-ABA moiety is formed from chorismate in plastids. Pteridine and p-ABA are then transported to the mitochondria, where they are coupled together, glutamylated and reduced to produce THF. A short chain of γ-linked glutamates can then be added in mitochondria, plastids or cytosol, yielding folate polyglutamates (THF-Glu_n). Folate molecules exist *in vivo* mainly as polyglutamates and these are preferred by folate-dependent enzymes involved in C_1-metabolism [15]. By contrast,

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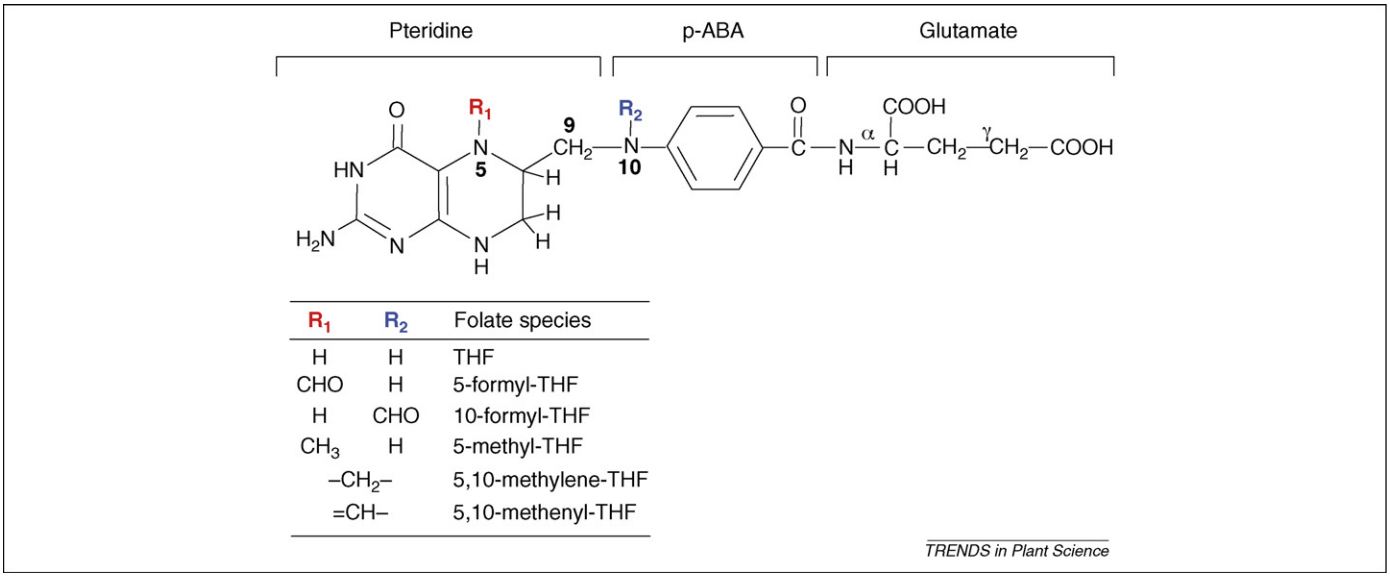

R_1	R_2	Folate species
H	H	THF
CHO	H	5-formyl-THF
H	CHO	10-formyl-THF
CH_3	H	5-methyl-THF
$-CH_2-$		5,10-methylene-THF
=CH–		5,10-methenyl-THF

Figure 1. Chemical structure of folates. The folate molecule consists of pterin, p-ABA and glutamate moieties marked with square brackets. The folate shown is the monoglutamyl form of tetrahydrofolate (THF). Plant folates have γ-linked polyglutamyl tails of up to approximately six residues attached to the first glutamate. C_1 units at various levels of oxidation can be attached to N-5 and/or N-10, as indicated by R_1 and R_2. The list of naturally occurring C_1 units is shown below the structural formula. The pteridine ring of folates can exist in tetrahydro, dihydro, or fully oxidized forms.

folate transporters typically prefer monoglutamates, thus glutamylation tends to favor folate retention within cells or compartments [16]. Folates are found in plant vacuoles, as well as in the cytosol, mitochondria and plastids [14,17], and can be taken up by plant cells from the culture medium [18]. Therefore, the intracellular distribution and localization of folates requires various transport steps (Figure 2). Most of these steps are likely to be carrier-mediated (as in other organisms), the exceptions being those involving p-ABA, which is able to cross membranes by diffusion because it is a hydrophobic weak acid [19]. However, the only plant folate transporters yet identified are both plastidial [20,21], thus at least three folate transporters (mitochondrial, vacuolar and plasmalemmal) and a mitochondrial pteridine transporter remain to be found (Figure 2).

Most folates are labile molecules. Thus, whereas detailed knowledge of folate biosynthesis in plants enables engineering of the pathway, the enhancement of the overall folate content to a level significantly impacting on human health also needs an in-depth understanding of folate degradation and salvage.

Folate breakdown and salvage

Folates undergo spontaneous oxidative or photo-oxidative cleavage of the C9–N10 bond (Figure 1) to give dihydropterin-6-aldehyde and *p*-aminobenzoylglutamate (p-ABA-Glu) fragments [22]. Folate breakdown can yield large folate losses in post-harvest fruits and vegetables [2]. For example, in peas 50% of the total folate at harvest was lost after six days of storage at ambient temperature. In other plant tissues, breakdown is apparently countered by salvage reactions that enable re-use of the breakdown products in folate synthesis [23]. These reactions hydrolyze p-ABA-Glu to yield p-ABA, and reduce the aldehyde group of dihydropterin-6-aldehyde to yield the folate synthesis intermediate hydroxymethyldihydropterin (HMDHP) (Figure 3) [23]. The aldehyde reduction is mediated by multiple, non-specific reductases, of which one has been cloned [24]. If the dihydropteridine ring becomes oxidized (which can occur spontaneously) before this reaction takes place, plants cannot reduce the aldehyde reduction product back to HMDHP and the pteridine is in effect lost [25]; this is also the case in *Escherichia coli* [25]. However, a reductase that catalyzes a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of oxidized pteridines is known in trypanosomatids [26]. Lack of such an enzyme, resulting in failure to reclaim oxidized pteridines, might, therefore, be seen as a 'weak point' in plant pteridine and folate metabolism.

Folates are protected from oxidative breakdown by binding to proteins [22,27], and, because polyglutamylation generally favors protein-binding [15], there is a positive correlation between polyglutamylation and folate stability. Protein-binding also protects polyglutamyl folates from deglutamylation by γ-glutamyl hydrolase [28]. However, these findings are based entirely on work in animals. Nothing is yet known about folate-binding proteins in plants, although the co-occurrence of polyglutamyl folates and high γ-glutamyl hydrolase activities in plant vacuoles [17] suggests that they exist.

Plants have developed mechanisms to cope with folate instability. Reducing folate degradation rate and salvaging its degradation products might contribute to folate

Table 1. Folate content of selected crops[a]

Crop	Folate content (μg 100 g^{-1})
Rice (white[b], raw)	6–8
Maize (yellow, seeds, raw)	19
Wheat (hard, white, raw)	38–43
Tomato (fruit, raw)	9–29
Peas (green, raw)	65
Spinach (leaves, raw)	194
Lentils (mature seeds, raw)	433
Beans (pink, mature seeds, raw)	463

[a]Values are in μg of folate per 100 g. Data are from the USDA National Nutrient Database for Standard Reference (http://www.nal.usda.gov/fnic/foodcomp/search/).
[b]Polished grains.

Box 1. Folate deficiency and health

Humans cannot synthesize folates (vitamin B9) and thus have to rely on plant food supplying these essential vitamins. The recommended dietary allowance (RDA) for an adult person is 400 μg and 600 μg for pregnant women (http://ods.od.nih.gov/factsheets/folate.asp).

Folate deficiency results in serious health problems, including megaloblastic anemia and neural tube defects (NTD), such as spina bifida and anencephaly. Adequate dietary folate intake can prevent these conditions [62]. Given that the neural tube is formed between days 21 and 27 after conception (before most women realize they are pregnant), NTD risk can only be minimized if women take high amounts of folate from the peri-conceptional phase until week 12 of gestation. Recent studies indicate that NTD incidence in the poorest regions in India and China can be up to 10 times higher than that in Western surveillance systems [63,64]. Low folate status is also associated with the occurrence of several neurodegenerative disorders (such as Alzheimer's disease) [65], a higher risk of cardiovascular disease [66] and development of a range of cancers [67], although no causal relationship has been established so far.

Folate deficiency first becomes visible in high-turnover cells, such as erythrocytes, resulting in megaloblastic anemia because of a deficit in DNA, necessary for normal cell division [2]. Other consequences are the induction of hyper-homocysteinemia, a risk factor for cardiovascular disease [66], misincorporation of uracil in DNA, and ultimately chromosomal breakage [68], resulting in cellular degeneration. Finally, folate deficiency leads to aberrant DNA-methylation patterns associated with carcinogenesis [67,69].

To reduce the risk of NTD, mandatory fortification of cereal-derived foods with synthetic folic acid has been implemented in the USA and other countries [70]. The amount of added folic acid is such that the predicted average intake resulting from consumption of fortified food products equals ~100 μg/day, corresponding to 170 μg dietary folate equivalents (DFE) because folic acid is assumed to be 1.7 times more bioavailable than natural folates [71]. Evaluation of efficacy showed that reality surpasses this prediction, and that the RDA was met or exceeded in most adults [72,73]. However, as excessive intakes of folic acid (>1 mg/day) might mask the diagnosis of vitamin B_{12} deficiency [74,75], fortification remains a controversial issue in the EU.

In third world countries, a solid infrastructural platform for effective population-based prevention in the form of fortification, supplementation or educational campaigns is lacking. Therefore, biofortification of staple crops used in concert with conventional public health practices will help in attaining the recommended dietary intake of folates, especially in developing countries.

enhancement. However, a greater insight into these mechanisms is needed to harness this knowledge into engineering strategies, which up until now have mainly concentrated on the engineering of the folate biosynthesis pathway.

Metabolic engineering of folate synthesis

GTP cyclohydrolase I (GTPCHI) overexpression

To date, all published work on enhancing plant or bacterial folate content has involved manipulating the activities of biosynthetic enzymes. Initial studies in tomato fruit and *Arabidopsis thaliana* [29,30] overexpressed the first enzyme of the pteridine branch of the folate pathway, GTP cyclohydrolase I (GTPCH I; Figure 2). In both cases, a non-plant gene [based on the mammalian or bacterial gene (GenBank accessions BE136861 and AE000304), respectively] was used, because the foreign enzyme was predicted be free of negative feedback control (inhibition by pathway products) *in planta*. Pteridine levels in transgenic tomatoes and *Arabidopsis*, respectively, rose to as much as 140- and 1250-fold those in wild-type controls; however, the rise in folate content was only two- to fourfold, indicating the need for further engineering of the pathway [29,30]. Analysis of total p-ABA (p-ABA plus its glucose ester) in transgenic tomato fruit indicated severe p-ABA depletion, and, consistent with this depletion, supplying exogenous p-ABA to GTPCHI-overexpressing transgenic tomatoes [29] and *Arabidopsis* [31] increased folate content by a further 2.5- to 10-fold. This observation points not only to the need for simultaneous enhancement of both folate precursors (pterin and p-ABA), but also proves a substantial physiological potential for increasing folate concentration within the plant cell.

Combined overexpression of GTPCHI with aminodeoxychorismate synthase (ADCS)

These promising findings prompted another round of engineering in tomato fruit in which the first enzyme of the p-ABA branch of the folate pathway, aminodeoxychorismate synthase (ADCS; Figure 2), was overexpressed, using the gene from *Arabidopsis* (At2g28880; GenBank accession NP_850127) [32]. The resulting transgenic fruit contained an average of 19-fold more p-ABA, compared with wild-type controls, without an increase in folate level. When this trait was combined with the pteridine-overproduction trait by crossing, the double transgenic fruit accumulated an average of 19-fold more folate than controls; the folate levels achieved (840 μg per 100 g edible portion) provide the complete adult daily requirement in less than one standard serving (1/2 cup). However, this engineering strategy also resulted in a 20-times higher accumulation of pteridines and p-ABA as compared with the wild-type control. Although the level reached for p-ABA in the transgenic tomatoes is harmless for human health, the situation for pteridines is unclear and needs investigation [32]. Humans and animals synthesize an important pterin, tetrahydrobiopterin (H4B), which participates in the synthesis of nitric oxide and neurotransmitters, such as dopamine (reviewed in [33,34]). In addition, dihydroneopterin and its oxidized form, neopterin, are well known markers of immune system stimulation and are widely used in diagnostics of numerous diseases [33]. They possibly also participate in the stress response [33,35]. Therefore, perturbing pteridine status might have consequences for human health, but relative roles and interactions of endogenously synthesized versus dietary pteridines in mammalian metabolism are unclear.

In an independent approach, folate biofortification of rice was recently achieved [36]. In this case, *Arabidopsis* genes encoding GTPCHI (Gene Bank accession AF489530) and ADCS were overexpressed under the control of strong endosperm-specific promoters on a single genetic locus. The presumably negatively feedback-regulated plant GTPCHI [37] was chosen to avoid an undesirably high accumulation of intermediates. Transgenic rice seeds overexpressing both *Arabidopsis* genes contained up to 100-fold higher folate levels as compared with the wild type (1723 versus 17 μg/100 g fresh weight). Cooking experiments have demonstrated that it is probable that 100 g of the biofortified rice grains can satisfy the daily folate requirement for an average adult person or at least supply most of it [36]. Moreover, the levels of the biosynthesis intermediates, pterins and

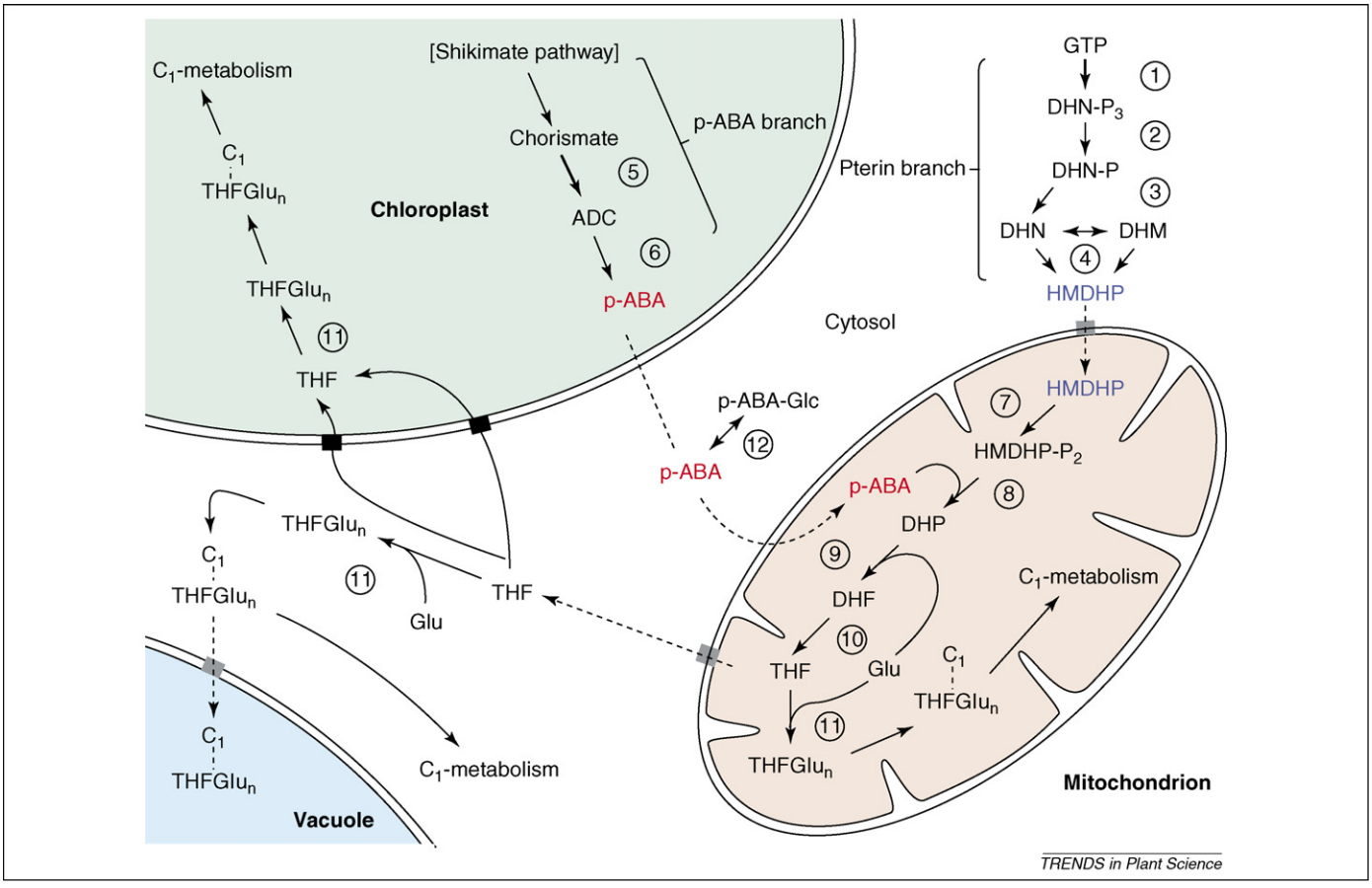

Figure 2. The folate biosynthesis pathway, its compartmentalization in plant cells and carrier-mediated transport steps. The two known folate carriers (both plastidial) are shown in black. Hypothetical carriers are shown in gray with dotted lines indicating hypothetical transport steps (the movement of p-ABA is most probably by diffusion). The hypothetical vacuolar folate carrier might transport polyglutamyl forms, unlike most other folate carriers. p-ABA occurs mainly as its glucose ester, which is formed in the cytosol via a reversible reaction with UDP-glucose [19]. Compound abbreviations: ADC, aminodeoxychorismate; DHF, dihydrofolate; DHM, dihydromonapterin; DHN, dihydroneopterin; DHP, dihydropteroate; –Glc, glucose ester; $–Glu_n$, polyglutamate; HMDHP, hydroxymethyldihydropterin; –P, phosphate; $–P_2$, diphosphate; $–P_3$, triphosphate; THF, tetrahydrofolate. Enzymes: 1, GTP cyclohydrolase I; 2, DHN-P_3 pyrophosphatase; 3, non-specific phosphatase; 4, dihydroneopterin aldolase (which mediates the epimerization of DHN to DHM, and the aldol cleavage of both); 5, aminodeoxychorismate synthase; 6, aminodeoxychorismate lyase; 7, hydroxymethyldihydropterin pyrophosphokinase; 8, dihydropteroate synthase; 9, dihydrofolate synthase; 10, dihydrofolate reductase; 11, folylpolyglutamate synthase; 12, p-ABA glucosyltransferase.

p-ABA, were substantially lower than in biofortified tomato fruit. The molar ratios of folates:p-ABA:pterins in folate-enhanced tomatoes are ~1:2.5:0.75 [32], whereas they are 1:0.5:0.013 in biofortified rice [36]. It is therefore tempting to speculate that the use of plant GTPCHI, which probably retains its intrinsic negative feedback regulation, in combination with plant ADCS, results in a balanced tuning of both enzyme activities, enabling a more optimal flux of pteridine precursors and p-ABA through the pathway.

The accumulated folates in double transgenic tomato fruit and rice grains showed normal proportions of C_1 forms but were not as extensively polyglutamylated as in controls [32,36]. A reduction in glutamylation has no negative impact on the nutritional value of folates and can even be beneficial by enhancing bioavailability, because a negative correlation with polyglutamate tail length has been demonstrated [38,39]. The accumulation of folate precursors in double transgenic tomato fruit, and to a lesser extent in transgenic rice grains, indicated a flux constraint at the downstream HMDHP-pyrophosphokinase (HPPK) step, suggesting the utility of a further round of engineering to boost the activity of this enzyme [32].

These two successful attempts at folate biofortification in a dicot and a monocot plant species demonstrate that the simultaneous enhancement of pterin and p-ABA branches can be used as a universal approach applicable to other plants. Optimization of metabolite fluxes can probably be achieved by engineering other pathway enzymes. Examples of such engineering in bacteria show a potential for application to plants.

Enhancing folates in bacteria

Increasing GTPCHI and HPPK activity by overexpression of *folKE* (a gene that encodes a bifunctional protein, displaying both aforementioned activities; GenBank accession number YP_809225) increased folate production threefold in *Lactococcus lactis* and, as in plants, reduced the extent of polyglutamylation [40,41]. Co-expressing folylpolyglutamyl synthase (GenBank accession YP_809228), which is responsible for adding a glutamyl-chain to THF, restored normal polyglutamylation. Overexpressing the downstream enzymes dihydropteroate synthase (DHPS) (GenBank accession YP_809226) and dihydrofolate reductase (DHFR) (GenBank accession YP_001033455) along with FolKE gave no additional increase in folate production, or even

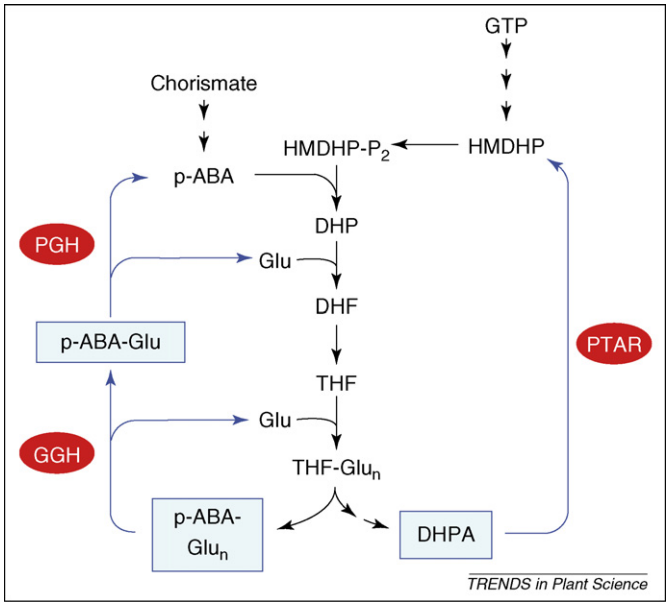

Figure 3. Folate salvage reactions (blue arrows) in relation to folate biosynthesis. Oxidative or photo-oxidative cleavage of folates gives rise to *p*-aminobenzoylglutamate (p-ABA-Glu) or its polyglutamyl forms (p-ABA-Glu_n) and a pterin aldehyde. In the case of tetrahydrofolate (THF), the aldehyde is tetrahydropterin-6-aldehyde, which can oxidize spontaneously to dihydropterin-6-aldehyde (DHPA). DHPA can also be formed directly from cleavage of dihydrofolate (DHF). Enzymes: GGH, γ-glutamyl hydrolase (which removes the polyglutamyl tail of p-ABA-Glu_n); PGH, p-ABA-Glu hydrolase; PTAR, pterin aldehyde reductase.

decreased production, as in the case of DHFR [40]. Taken together, these results warrant trials for engineering HPPK activity in plants because the effects of engineering HPPK as well as GTPCHI were not separated in *L. lactis* and might have been additive. A more theoretical approach to folate pathway engineering in *Bacillus subtilis* achieved an eight-fold increase in folate production by amplifying expression of the genes in a folate operon, combined with genetic manipulations designed to increase p-ABA formation [42].

Other metabolic engineering strategies

Several ways to engineer folate content besides overexpressing biosynthesis genes potentially exist. Leaf vacuoles contain folates that are probably protein-bound [17], so if vacuolar transporter and folate-binding proteins (FBPs) had been identified (which is not yet the case), either or both might be manipulated to enhance storage in vacuoles, in which folates are able to accumulate to high levels [17]. For grains or other storage organs, a plant FBP (if these exist) or the well characterized bovine milk FBP [27] might be targeted to protein-storage vacuoles. Tightly sequestering the end-products of the pathway in this manner could bring about a folate-sink effect, increasing biosynthetic flux by releasing negative-feedback controls and causing precursor pools to shrink rather than expand. Another strategy for fruits and vegetables would be to cut post-harvest losses [2,23]. To do this, we would first need to know (which we do not) whether the net folate breakdown reflects increased degradation, or decreased salvage or synthesis. When both p-ABA and pteridines were overproduced in tomato, it was observed that pteridines were largely oxidized after ripening [29], in which state they can no longer be used for folate synthesis. Introducing a foreign (and potentially feedback-free) pteridine reductase [26] might thus be helpful to boost folate salvage capacity.

A more readily implemented strategy is to reduce expression of the enzyme that returns 5-formyl-THF (a stable folate that is not a C_1 donor) to the active C_1 folate pool [2]. This should cause build-up of 5-formyl-THF, and probably of total folate, and indeed the first experiments exploring this path showed 5-formyl-THF accumulation and a doubling of total folate levels [43]. However, 5-formyl-THF accumulation is a double-edged sword: although this folate is not a C_1 donor, it inhibits folate-dependent enzymes and hence can disrupt C_1 metabolism [43]. Despite observations that some over-accumulation of 5-formyl-THF (using 10mM 5-formyl-THF in feeding trials on *Arabidopsis* plants) is well tolerated [43], these inhibitory effects most probably limit the extent to which 5-formyl-THF can accumulate in the cytosol, mitochondria or plastids (but not vacuoles, which contain no folate-dependent enzymes to inhibit).

Parallel to using transgenic technology to enhance folate levels in plants, it is important that the pursuit of classical breeding strategies is continued, because products emerging from this approach are readily accepted by consumers.

Natural variation in folate levels

Exploiting natural genetic variation within species and between related species is a paradigm for crop improvement, and combining genomics with conventional breeding methods has an enormous potential for nutritional improvement of crops [9–11]. Varieties can be phenotypically analyzed on a large scale as soon as high-throughput procedures for folate determination are available [44]. This should enable mapping of quantitative trait loci (QTLs), ultimately to be integrated in molecular marker-assisted selection. Crops such as rice, maize and wheat are appealing targets for enrichment, because they contain low levels of folates and are staple foods in developing countries. Given their low intrinsic folate concentrations, it might be the case that only limited enhancement can be achieved through breeding strategies [45]. However, this has yet to be established, so it is important to explore this approach. Significant opportunities exist to enhance folate in tomato where wild genetic resources are being harnessed through conventional breeding. This goal is now a realistic possibility because of the availability of well characterized interspecific introgression lines (ILs) and novel populations. Introgression populations are available, which are comprised of marker-defined regions of wild-species genomes introgressed into a *Solanum lycopersicum* background [46]. Preliminary studies employing the microbiological assay to screen ILs have observed several-fold variation in folate abundance within the population (G. Tucker, personal communication). These near isogenic lines are a powerful tool for fine mapping of QTLs [47]. Moreover, mapped QTLs are potentially valuable in identifying and eventually cloning new target genes involved in folate metabolism and its regulation. The availability of complete genome sequence information and a large array of molecular markers make a QTL approach particularly feasible in rice [48].

Enhancing folate bioavailability

Bioavailability issues will have to be addressed regardless of what method is being applied, because poor bioavailability could annihilate the effect of enhanced folate content; conversely, improving bioavailability could increase the nutritional value of a crop without the need to enhance nutrient levels as such. Natural folates exhibit reduced bioavailability compared with synthetic forms. It is known that polyglutamylation can reduce folate bioavailability, because dietary folates need to be deglutamylated by the intestinal conjugase, an enzyme that hydrolyses the polyglutamate tail, before efficient intestinal uptake can take place [45]. Recently, however, it has been shown that the ratio of monoglutamate to polyglutamate in natural food folate-derivatives had no apparent influence on the intestinal absorption [38], suggesting that the amount of the intestinal conjugase is more than adequate to remove the polyglutamate tail without affecting the absorption rate. This suggests that other factors have an impact on bioavailability, such as entrapment of folates in the food matrix, rendering them inaccessible to the conjugase that is tethered to the intestinal cell membranes. Therefore, one potential strategy to increase bioavailability could be to enhance levels of the plant conjugase activity of gamma-glutamyl hydrolase (GGH), which would be released from the vacuole following maceration and facilitate folate release within the food matrix before digestion. An alternative strategy is the use of FBPs. A hitherto unidentified component of cow's milk, possibly FBP, has been reported to improve the bioavailability of food folates [49], but the relevance to human nutrition of the study on which this conclusion was based has been questioned. One must also consider the fact that the presence of FBPs in the food matrix, although mechanistically unclear, can lead to a reduction in folate absorption [50]. Nutritional studies might in this way present new approaches that could be implemented in the creation of a health beneficial crop. Determination of folate bioavailability and bioefficacy in such a crop will ultimately require data from controlled absorption tests in human volunteers, and data from larger feeding trials in folate-deficient test populations will have to be acquired to demonstrate the efficacy of enriched crop varieties. In addition, there will be a need to assess whether high pterin or p-ABA concentrations accumulated as a result of engineering have any effect on folate uptake, transport or metabolism [51].

Finally, it should be mentioned that the water-soluble nature of the folate cofactor removes a major criticism [52] that has been leveled at provitamin A-enriched rice grains [53]. Because provitamin A is fat-soluble, adequate intake of fats is essential for its absorption, which is not guaranteed in the diet of the poor. This is not a problem for folates.

The feasibility of folate-biofortified crops

Direct advantages of biofortification

Using a folate-enriched staple crop to help combat folate deficiency has two immediate advantages. First, biofortification offers sustainability when compared with industrial fortification (addition of synthetic folic acid to cereal-derived foods) and pharmaceutical supplementation (Box 1). Development of a biofortified crop is largely a one-time investment that can benefit the health of millions and therefore establishes a multiplier effect [54]. Second, biofortified crops can improve folate intake by malnourished rural populations that are unlikely to benefit from commercially fortified foods or supplements, which are often only available in cities. Therefore, biofortification is complementary to these other intervention strategies.

Regional adaptation

To ensure the adoption of biofortification by farmers, it is important that crop productivity and/or profits are increased simultaneously. This can be accomplished by introducing the biofortification trait into high-yielding (elite) genotypes [55]. Therefore, biofortified lines will have to enter breeding programs to be crossed with varieties that are locally adapted to a given agro-ecosystem. This will evidently require a certain level of national research capacity [56]. However, in regions such as South-East Asia, effective systems for disseminating improved crop varieties are already in place, making such implementation costs minimal [57].

Cost-effectiveness of biofortification

Biofortification is expected to be cost-effective based on studies quantifying the potential health benefits for vitamin A, iron and zinc-enhanced crops using the disability-adjusted life years (DALY) approach [57,58]. This model calculates the reduction of the current disease burden associated with vitamin deficiency (i.e. the mortality and morbidity burden quantified as the number of DALYs lost) resulting from biofortification, taking into account current intake of the staple crop, the expected intake of micronutrient(s) after crop biofortification, and the percentage of the population expected to consume the biofortified crop. Application of the DALY methodology to the impact of golden rice 2 (an improved version of β-carotene biofortified golden rice) in India gave an estimated reduction of the burden of vitamin A deficiency (VAD) of between 9% (low-impact scenario) and 59% (high-impact scenario) [59]. Even the 9% reduction translates into saving many thousands of people from blindness or death from infectious disease. Moreover, golden rice 2 promises to be cost effective, because it was estimated that even under a low impact scenario the cost of saving one DALY is less than US$20 as compared with a cost of at least US$134 of doing it by vitamin A supplementation [59].

Towards consumer acceptance

It is unlikely that folate-enrichment of food plants will result in any sensory change, as is the case for golden rice, so from this standpoint consumer acceptance should not be compromised. However, concerns about potentially harmful environmental (e.g. loss of biodiversity) and health (e.g. allergy) impacts are inevitable, especially in the European Union, particularly if biofortification is achieved by metabolic engineering. This does not mean that engineering approaches should be abandoned, because acceptance of 'genetically modified' crops is likely to grow as their benefits become clear to consumers [60], as has happened for other scientific and medical breakthroughs of the past two centuries (e.g. the widespread controversy around the

first mass-vaccination, an intervention that ended up eradicating smallpox, at that time a deadly disease). The health risks and benefits of genetic manipulation of food crops depend almost exclusively on the chemical composition (profiles of nutrients, metabolites, antinutritional factors, etc.) of the resulting product, not on the technology used to achieve the modification [61]. Indeed, it can be argued that genetic engineering provides more targeted, specific and predictable alterations of food crop composition than occurs with the various 'conventional' approaches (including conventional breeding, mutation breeding, somaclonal variation, somatic hybridization, etc.) [61]. Moreover, much can be learned from metabolic engineering studies about how folate content is controlled in plants, and this knowledge suggests how folate levels can be increased without using transgenic technology.

Conclusion

Folate-biofortified rice, tomatoes and *Arabidopsis* plants have already been developed using simple metabolic engineering stratagems, and enough is known about plant folate biochemistry to envisage other biofortified crops, such as wheat, banana and potato. Currently, folate deficiency persists, and will continue to do so until we deploy agriculture-based strategies to help reduce this global burden. Folate biofortification of staple crops should be a valuable, complementary and cost-effective intervention in fighting folate deficiency worldwide, above all in poor countries.

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