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Folate biofortification in food crops

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Folates are essential vitamins in the human diet. Folate deficiency is still very common, provoking disorders such as birth defects and anemia. Biofortification via metabolic engineering is a proven powerful means to alleviate folate malnutrition. A variety of metabolic engineering approaches have been successfully implemented in different crops and tissues. Furthermore, ensuring folate stability is crucial for long-term storage of crop products. However, the current strategies, shown to be successful in rice and tomato, will need to be fine-tuned to enable adequate biofortification of other staples such as potato, wheat and cassava. Thus, there is a need to overcome remaining hurdles in folate biofortification. Overall, biofortification, via breeding or metabolic engineering, will be imperative to effectively combat folate deficiency.

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Folates are a group of water soluble B-vitamins (vitB9), consisting of a pteridine ring, a para-aminobenzoate moiety (*p*-ABA) and a γ-linked tail with one or more L-glutamates (Figure 1) [1]. Folates are labile compounds, prone to (photo-)oxidative cleavage [2]. Specific folate entities are chemically distinguished by three different structural modifications. First, folates exist in varying oxidation states, with tetrahydrofolate (THF) being the most reduced form. THF is the bioactive vitamin, functioning as an essential co-enzyme in numerous metabolic reactions. Second, folates can harbor a range of one-carbon (C1) units on the pteridine (N5) and *p*-ABA (N10) moiety, influencing their stability. Third, the length of the glutamate tail is highly variable [3]. A longer glutamate tail facilitates binding of the vitamin to folate-dependent enzymes, as well as ensuring its cellular retention [4]. Polyglutamylated folates can therefore be considered more stable than monoglutamates *in vivo*.

The chemical diversity of folates reflects a perfect adaptation to their varied biological function as C1-donors and acceptors, rendering them a pivotal role in primary metabolism of nearly all organisms. Folate-dependent enzymes play a key role in thymidylate and purine synthesis, as well as pantothenate (vitB5) formation [3]. 5-methyl-THF donates its methyl group to homocysteine to form methionine by the action of the cobalamin (vitB12)-dependent methionine synthase [5]. In plants, folates have an additional essential role in photorespiration, as well as in chlorophyll, plastoquinone, tocopherol, pectin and lignin synthesis [6].

Due to their central role in primary metabolism, detrimental physiological effects arise upon folate deficiency [7]. Animals, unable to synthesize folates *de novo*, rely primarily on their diet for an adequate folate supply. Decreased folate levels result in impeded erythrocyte development, causing megaloblastic anemia. During embryogenesis, folate deficiency provokes aberrant neurulation, leading to the onset of neurodegenerative disorders such as anencephaly and spina bifida [8]. Together, folate deficiency induced Neural Tube Defects (NTDs) are estimated to account for over 150 000 birth defects each year, predominantly in the developing world [9].

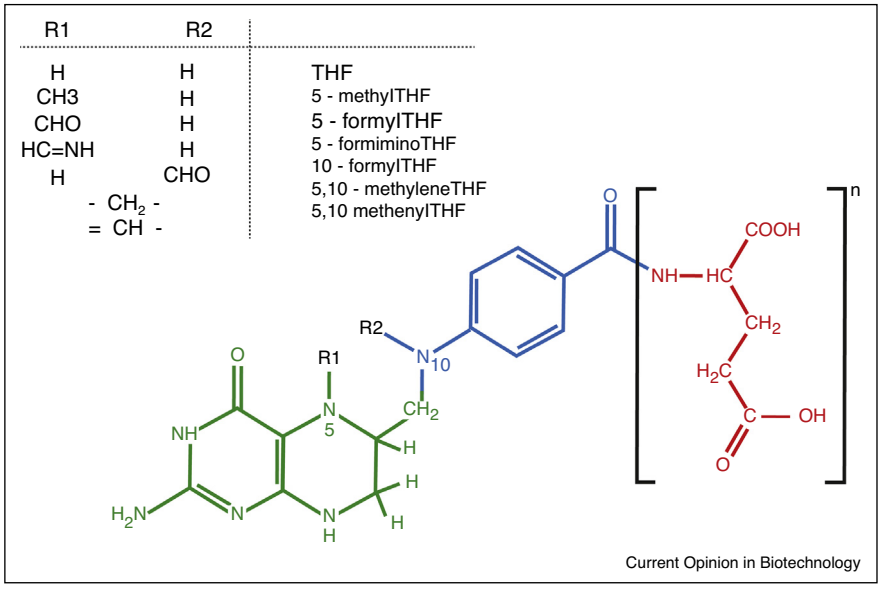
Fermented foods, leguminous and leafy vegetables can be considered rich sources of folates. However, some massively consumed staple crops, such as rice, corn, wheat, potato and cassava, contain inadequate folate levels (Table 1). The recommended daily intake (RDI) of folate is 400 μg for an adult, increasing to 600 μg during pregnancy [4]. Unfortunately, many diets, in developing as well as developed countries, fail to reach these standards.

A combined strategy of technical, socio-economical and biotechnological solutions will be essential to relieve this global burden. In this review, current state-of-the-art on biotechnological approaches for folate biofortification will be discussed.

Biosynthesis

In plants, folate biosynthesis is characterized by subcellular compartmentation (Figure 2) [3]. The pterin branch of folate biosynthesis takes place in the cytosol, yielding 6-hydroxymethyldihydropterin (HMDHP). Secondly,

Figure 1



Chemical structure of folates.
Folates consist of three moieties: a pteridine (green), a *p*-aminobenzoate molecule (blue) and a glutamate tail (red). The green/blue transition reflects a (photo)-oxidation-labile bond. The folate shown is a polyglutamylated tetrahydrofolate (THF). Plant folates carry up to eight glutamates [4,67]. Different folate forms are distinguished by different C1-substituents, at different levels of oxidation, on N5 or N10.

the *p*-ABA branch resides in plastids, consuming chorismate as a substrate. The resulting *p*-ABA, together with HMDHP, are assumed to enter the mitochondria by passive diffusion and carrier mediated transport, respectively [2]. Condensation of the two moieties occurs in mitochondria, followed by polyglutamylation of the resulting folate [10]. However, polyglutamated folates are retained in mitochondria, as they are intracellularly transported as monoglutamates, with vacuolar import being the only known exception [11].

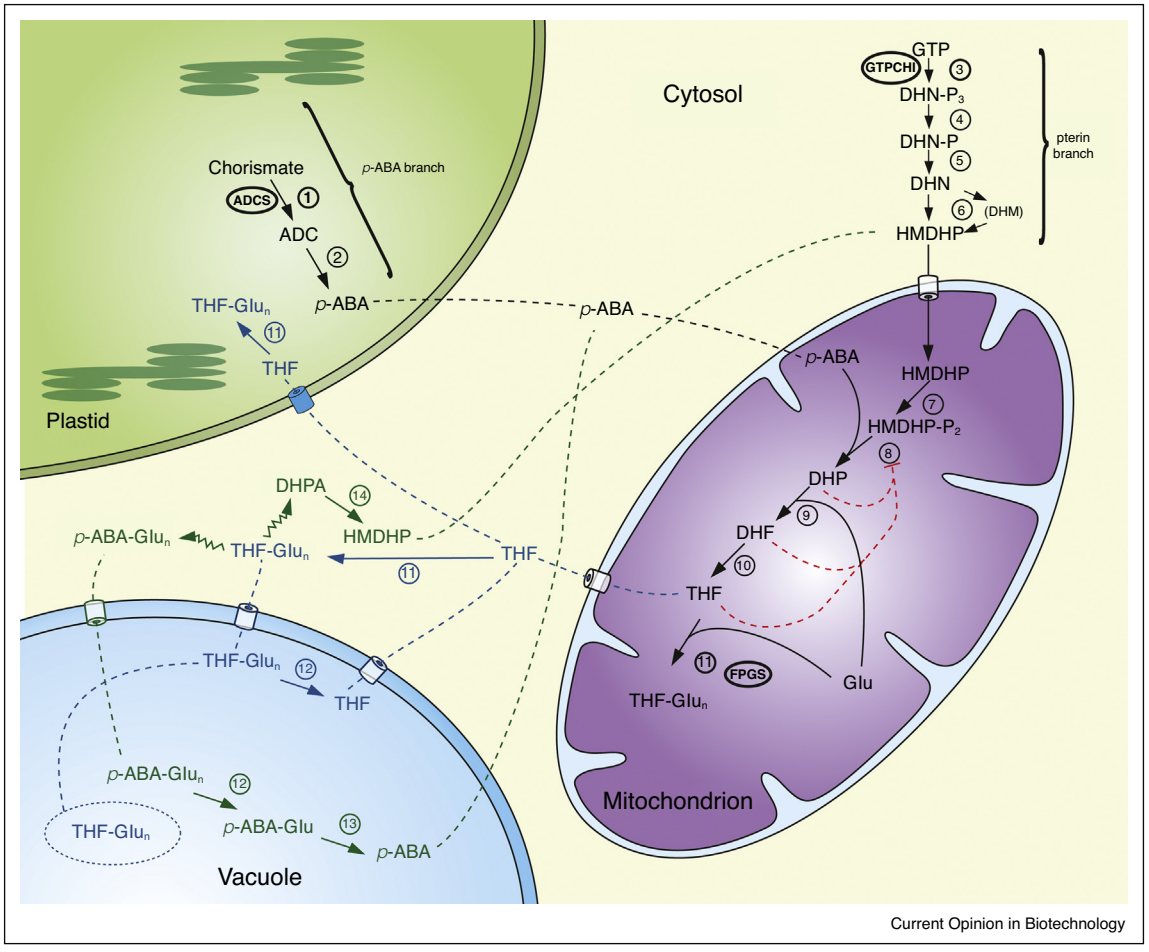
Table 1

Folates in foods. Different food products are ranked according to folate content. Most staples contain inadequate levels of folate (RDI: 400 μg for adults; 600 μg for pregnant women). Brie cheese is an example of a fermented food. Data on folate content were derived from the USDA National Nutrient Database for Standard Reference (Release 28, September 2015, revised in May 2016). Based on these data, the fold increase, needed to obtain a sufficient amount of folate to reach the RDI in 100 g of raw food material, was calculated. As adequate folate levels are most critical during pregnancy, 600 μg was set as the target level. Possible losses during processing and variation of folate bioavailability – both shown to notably decrease the amount of bioeffective folate, as for instance in rice endosperm [35••,69] – are not accounted for)

Food	Folate content (μg/100 g)	Fold increase to reach RDI in 100 g	Global supply[a] (g/capita.day)
Rice, white, long-grain, regular, raw	8	75	148.2
Tomatoes, red, ripe, raw	15	40	55.4
Potatoes, flesh and skin, raw	15	40	94.9
Corn grain, yellow	19	32	48.2
Plantains, raw	22	27	9.6
Cassava, raw	27	22	40.3
Lettuce, green leaf, raw	38	16	/
Wheat, soft white	41	15	178.8
Cheese, Brie	65	9	/
Spinach, raw	194	3	/
Beans, white, mature seeds, raw	388	2	6.8
Lentils, raw	479	2	/
Turkey, liver, raw	677	–	/

[a] Data on average global supply (if available) of the corresponding (wet) crop product are derived from FAOSTAT, 2011 (http://faostat.fao.org)(Food Supply—Crops Primary Equivalent). For rice, milled equivalent is presented.

Figure 2



Overview of folate biosynthesis and salvage.
Biosynthesis (black). In plastids, para-aminobenzoate (*p*-ABA) is synthesized from chorismate, originating from the shikimate pathway. The first step in the plastidial *p*-ABA branch of folate biosynthesis is aminodeoxychorismate (ADC) formation by the action of ADC synthase (ADCS). In the cytosol, 6-hydroxymethyldihydropterin (HMDHP) is formed (pterin branch), the first step of which is the conversion of guanosine triphosphate (GTP) to dihydroneopterin triphosphate (DHN-P_3) by GTP cyclohydrolase I (GTPCHI). Pterin and *p*-ABA moieties are condensed and reduced in the mitochondria. The final step of folate biosynthesis is polyglutamylation of tetrahydrofolate (THF) by folylpolyglutamate synthetase (FPGS) to folate polyglutamates (THF-Glu_n). Genes applied in successful biofortification approaches are indicated in **bold and encircled**. **Salvage (green)**. Serrated arrows symbolize (photo-) oxidative cleavage of folates. Dihydropterin-6-aldehyde (DHPA) and *p*-aminobenzoyl-polyglutamate (*p*-ABA-Glu_n) originate from (photo-)oxidative cleavage of folates and require salvage reactions to ensure recycling of folate biosynthetic intermediates. HMDHP is recovered by pterin aldehyde reductase (PTAR), reducing DHPA. Two subsequent deglutamylation reactions in the vacuole convert *p*-ABA-Glu_n to *p*-ABA, the first of which is catalyzed by γ-glutamyl hydrolase (GGH), yielding *p*-ABA-Glu. The remaining glutamate residue is removed by *p*-ABA-Glu hydrolase (PGH). **Transport and storage (blue).** THF is able to exit the mitochondrion, where it can be polyglutamylated by cytosolic FPGS (ctFPGS). Plastids can take up THF from the cytosol, followed by their polyglutamylation by plastidial FPGS. THF-Glu_n are able to enter the vacuole where they can be converted to THF by GGH, or retained in a storage form (dotted ellipse) [11]. THF-Glu_n serve as a cofactor in one-carbon metabolism in different subcellular compartments. Barrels represent transporter proteins, corresponding with known (solid) or unknown (transparent) genes. **Regulation (red).** Dihydropteroate synthase (DHPS) is known to be feedback inhibited by the three subsequent folate biosynthesis intermediates [68]. **Abbreviations**. DHN-P, dihydroneopterin monophosphate; DHN, dihydroneopterin; DHM, dihydromonapterin; HMDHP-P_2, 6-hydroxymethyldihydropterin pyrophosphate; DHP, dihydropteroate; DHF, dihydrofolate; Glu, glutamate. **Enzymes**. 1, ADC synthase (ADCS); 2, ADC lyase; 3, GTP cyclohydrolase I (GTPCHI); 4, DHN-P_3pyrophosphatase; 5, non-specific phosphatase; 6, DHN aldolase; 7,HMDHP pyrophoshokinase; 8, DHPsynthase; 9, DHFsynthetase; 10, DHFreductase; 11, folylpolyglutamate synthetase (FPGS); 12, γ-glutamyl hydrolase (GGH); 13, *p*-ABA-Glu hydrolase (PGH); 14, pterin aldehyde reductase (PTAR).

Salvage

Plants require salvage reactions to enable recycling of dihydropterin-6-aldehyde (DHPA) and *p*-aminobenzoyl-(poly)glutamate (*p*-ABA-Glu_n), originating from oxidative cleavage of folates. DHPA is reduced in the cytosol to retrieve HMDHP [12]. When fully oxidized, however,

pterins can no longer be recycled into folate [13]. To allow salvage of *p*-ABA, the glutamate tail is removed in the vacuole.

Fighting folate deficiency

A varied diet containing folate-rich foods is the optimal approach in combating folate deficiency. However, this requires global educational efforts combined with dietary interventions. As an alternative, application of the synthetic folate analog, folic acid, via supplementation or fortification of processed foods such as flour, has been implemented in many countries [14]. Although health benefits of folic acid fortification on NTD incidence stand undisputed [15,16], adverse effects may emerge upon high intake. Excessive supplementation results in high levels of unmetabolized folic acid in blood plasma, which has been linked to an increased risk of colorectal cancer in men [17] and impaired immunity in women [18]. Therefore, supplementation of synthetic folic acid, although proven to be very successful, should be dealt with caution [18–20]. In addition, the use of probiotic gut bacteria, overproducing folates, has been suggested [21]. These interventions are, however, difficult to implement in poor rural regions, where they are most needed [4]. Fortunately, biofortification (the enhancement of the natural folate content in crops) promises to be a cost-effective complementary strategy in the battle against micronutrient malnutrition [22•].

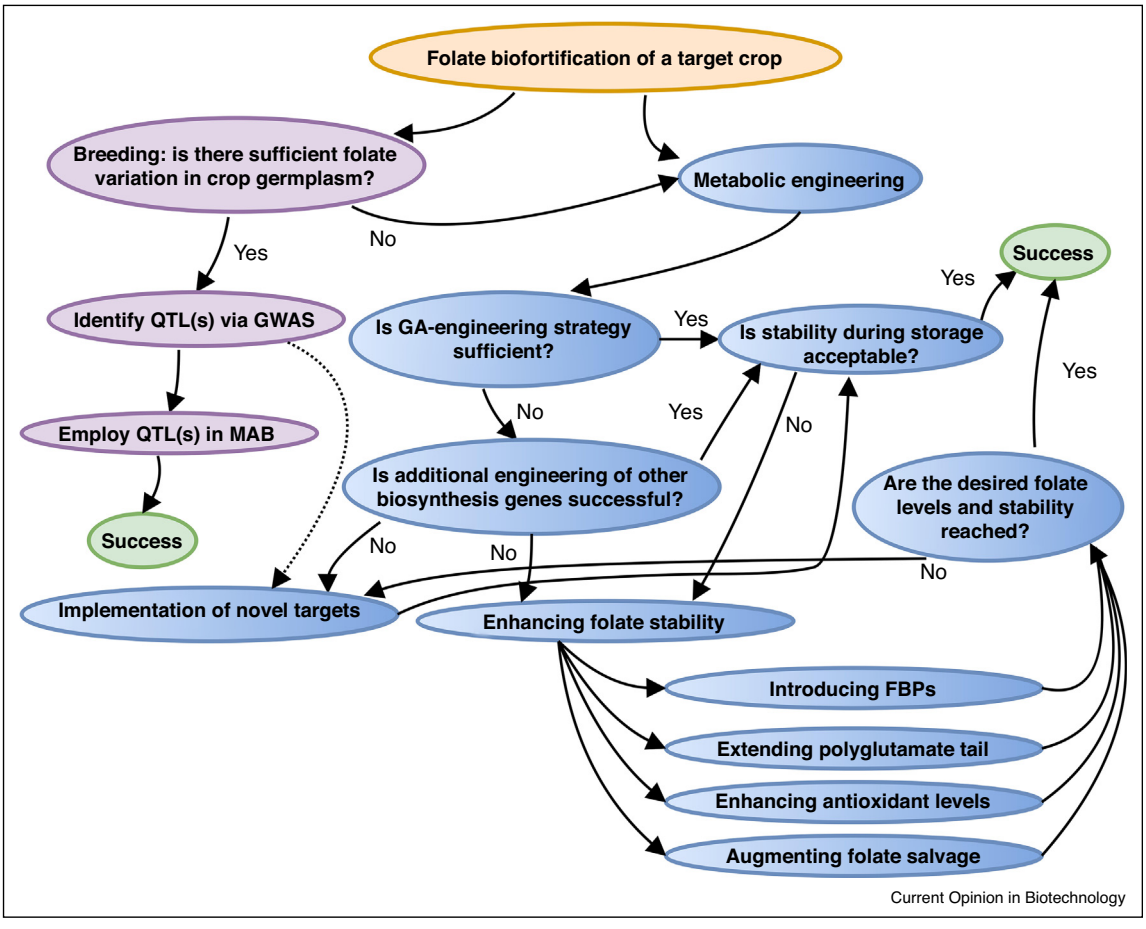
Advances in folate biofortification

A flow-chart of different approaches towards biofortification is presented in Figure 3.

Breeding

Conventional breeding for nutritional enhancement relies on inheritance of favorable quantitative trait loci

Figure 3



Flow-chart of possible approaches towards folate biofortification.
The flowchart indicates the different requirements that need to be assessed in the process towards biofortification of a target crop, as well as the order in which these should be addressed. Ellipses represent steps in biofortification via breeding (purple) or metabolic engineering (blue) towards a successfully biofortified target crop/tissue (green). Dotted arrow indicates the possible implementation of targets, identified via QTL annotation, in metabolic engineering approaches.

(QTLs) from sexually compatible parental lines. Therefore, biofortification by breeding is constrained by the natural variation of the desired trait present in the available collection of crop germplasm, as well as by being time-consuming [23]. However, genome-wide association studies (GWAS) combined with marker assisted breeding (MAB) prove to be a powerful tool in biofortification, demonstrated by the identification of multiple maize QTLs responsible for 3.22 and 5.76-fold increase in β-carotene (provitamin A) and α-tocopherol (vitamin E) content, respectively [24,25]. Although previous studies suggested insufficient folate variation in rice and wheat accessions [3,26], screening vast collections of germplasm might reveal greater diversity and thereby favor the applicability of breeding strategies [27]. Indeed, by investigating 78 rice germplasms, up to 7.6-fold difference in milled rice folate content was observed [28]. Similarly, considerable variation in folate levels was found in different potato, spinach and dry bean accessions [29•,30,31]. The availability of germplasm containing sufficient folate variation has enabled identification of three rice QTLs, influencing grain folate content [32•]. The identified rice QTLs do not correspond to folate biosynthesis genes, which are known to play a decisive role in folate accumulation [33,34,35••]. Interestingly, three rice genes were attributed to one of these QTLs [32•]. These include a rice gene, homologous to human folate hydrolase (catalyzing the shortening of the glutamate tail), as well as a homolog of an *Arabidopsis* plastidial folate transporter and a serine hydroxymethyl transferase. This indicates that QTL-mapping, apart from its high potency to be used in marker-assisted breeding (MAB), enables assignment of certain genes to be implicated in folate accumulation and possible discovery of novel factors in folate metabolism (Figure 3).

Metabolic engineering

Boosting folate biosynthesis

Boosting folate biosynthesis via metabolic engineering was the first proposed strategy to biofortify plants [36–38], as it had proven its potency in lactic acid bacteria [39]. This was first assessed in G-engineered *Arabidopsis*, where heterologous expression of a bacterial GTP cyclohydrolase I (*GTPCHI*), the enzyme executing the first committed step in cytosolic pterin synthesis (push-strategy), yielded up to a fourfold increase in total folate content [36]. The sole introduction of *GTPCHI* has resulted in a similar level of enhancement in tomato, maize, lettuce and Mexican common bean [37,40,41,42••] (Table 2). The modest enrichment of folate levels by this strategy (up to ninefold), together with the strong accumulation of pterin precursors [36,37,42••], suggested the existence of an additional bottleneck in folate biosynthesis [43•]. This is probably the consequence of a depleted *p*-ABA pool, as feeding the transgenic plants with *p*-ABA resulted in additional folate enhancement [37,42••]. The ability of a depleted *p*-ABA pool to constrain folate accumulation is further highlighted in rice by combining expression of different folate biosynthesis genes (except *ADCS*) with *GTPCHI*, rendering no further improvement compared to the G-engineered parental lines [44•]. Surprisingly, the *p*-ABA levels in the engineered Mexican common bean lines were elevated, though still shown to limit folate accumulation. This phenomenon has not been detected in previous G-engineered crops and reveals the possible existence of a feedforward regulatory mechanism.

Likewise, single gene approaches using aminodeoxychorismate synthase (ADCS), performing the first step towards *p*-ABA formation in plastids, resulted in a decrease or insignificant enhancement of folate levels in rice and potato [33,45]. Furthermore, an assessment of different one-gene approaches in rice endosperm revealed that ectopic expression of dihydrofolate synthase (*DHFS*) and folylpolyglutamate synthase (*FPGS*), which represent two of the three final mitochondrial folate biosynthesis steps (pull-strategy), ensure a very modest increase in folate content [44•]. The same study was unable to confirm previously reported 1.4-fold folate enhancement in rice seeds by the sole introduction of HMDHP pyrophosphokinase/dihydropteroate synthase (*HPPK/DHPS*) [46], catalyzing the first steps in mitochondrial folate biosynthesis. These findings reveal that single gene approaches will likely remain insufficient for high folate accumulation and indicate the need for multi-gene strategies.

Indeed, combined usage of *GTPCHI* and *ADCS* transgenes, thereby stimulating both pterin and *p*-ABA branches of folate biosynthesis, enables folate overproduction in tomato fruit and rice endosperm, reaching the desired target levels [33,47]. However, the enrichment of folates by this *GTPCHI/ADCS* (GA)-strategy results in a shift of the polyglutamylation ratio, as monoglutamates are more prevalent. Despite the success of the GA-strategy in tomato and rice, extrapolation towards biofortification of *Arabidopsis* or potato tubers appeared ineffective [45]. The increased levels of pterins and *p*-ABA in these GA-engineered plants indicated the presence of an additional restriction in folate biosynthesis, possibly linked to more strict regulation in meristematic tissues [4]. Moreover, accumulation of tetrahydrofolates in rice endosperm is proposed to be solely restricted by GTPCHI and ADCS activity, since basal expression of the endogenous folate biosynthesis genes remain unaltered in GA-engineered rice [34]. Conversely, endogenous folate biosynthesis is upregulated in GA-tomato [48].

Boosting folate stability

Folate stability, though often neglected, is problematic, as folate levels drop more than 50% during 4 month storage of GA-engineered rice grains [35••]. Moreover,

Table 2

Overview of metabolic engineering strategies to enhance folate levels in plants

Engineering approach	Genes engineered	Gene origin	Target species	Folate change	Reference
Biosynthesis	*ADCS*	*Arabidopsis thaliana*	Rice seeds	(1/6)-fold	Storozhenko *et al.* [33]
Single-gene	*ADCS*	*Arabidopsis thaliana*	Potato	insignificant	Blancquaert *et al.* [45]
	GTPCHI	*Escherichia coli*	*Arabidopsis*	fourfold	Hossain *et al.* [36]
	GTPCHI	*Arabidopsis thaliana*	*Arabidopsis*	insignificant	Blancquaert *et al.* [45]
	GTPCHI	*Mus musculus*	Tomato	twofold	de la Garza *et al.* [37]
	GTPCHI	*Arabidopsis thaliana*	Potato	twofold	Blancquaert *et al.* [45]
	GTPCHI	*Escherichia coli*	Maize	twofold	Naqvi *et al.* [40]
	GTPCHI	*Gallus gallus*	Lettuce	ninefold	Nunes *et al.* [41]
	GTPCHI	*Arabidopsis thaliana*	Mexican common bean	threefold	Ramírez Rivera *et al.* [42••]
	GTPCHI	*Arabidopsis thaliana*	Rice seeds	insignificant	Storozhenko *et al.* [33]
	GTPCHI	*Arabidopsis thaliana*	Rice seeds	6.1-fold	Dong *et al.* [44•]
	HPPK/DHPS	*Triticum aestivum*	Rice leaves	twofold	Gillies *et al.* [46]
	HPPK/DHPS	*Triticum aestivum*	Rice seeds	1.4-fold	Gillies *et al.* [46]
	HPPK/DHPS	*Arabidopsis thaliana*	Rice seeds	insignificant	Dong *et al.* [44•]
	DHFS	*Arabidopsis thaliana*	Rice seeds	1.27-fold	Dong *et al.* [44•]
Multi-gene	*GTPCHI* + other biosynthesis genes[a]	*Arabidopsis thaliana*	Rice seeds	6.1-fold	Dong *et al.* [44•]
	GTPCHI + *ADCS*	*Arabidopsis thaliana*	Rice seeds	100-fold	Storozhenko *et al.* [33]
	GTPCHI + *ADCS*	*Arabidopsis thaliana*	*Arabidopsis*	insignificant	Blancquaert *et al.* [45]
	GTPCHI + *ADCS*	*Mus musculus* (*GTPCHI*), *Arabidopsis thaliana* (*ADCS*)	Tomato	25-fold	de la Garza *et al.* [47]
	GTPCHI + *ADCS*	*Arabidopsis thaliana*	Potato	threefold	Blancquaert *et al.* [45]
Polyglutamylation	*FPGS*	*Arabidopsis thaliana*	Rice seeds	1.45-fold	Dong *et al.* [44•]
	FPGS	*Oryza sativa*	Rice seeds	4.7-fold	Abilgos Ramos, 2010[b]
	GTPCHI + *ADCS* + *FPGS*	*Arabidopsis thaliana*	Rice seeds	100-fold	Blancquaert *et al.* [35••]
Folate binding proteins	*FBP*	*Bos taurus*	Rice seeds	6.2-fold	Abilgos Ramos, 2010[b]
	GNMT	*Rattus norvegicus*	Rice seeds	8.8-fold	Abilgos Ramos, 2010[b]
	GTPCHI + *ADCS* + *FBP*	*Arabidopsis thaliana* (*G* + *A*) *Bos taurus* (*FBP*)	Rice seeds	150-fold	Blancquaert *et al.* [35••]
Homeostasis	*GGH* RNAi	/	*Arabidopsis*	1.3-fold	Akhtar *et al.* [11]
	5-FCL ablation	/	*Arabidopsis*	twofold	Goyer *et al.* [51]

[a] A two-gene approach was conducted, combining *GTPCHI* with respectively *ADCL*, *DHNA*, *HPPK/DHPS*, *DHFS*, *DHFR* and *FPGS*.
[b] Abilgos Ramos R, PhD thesis, University of Nottingham, 2010.

as folates are labile compounds, enhancing their stability could further boost folate build-up (Figure 3).

Polyglutamylation

Polyglutamylation positively influencing folate stability, together with the observed elevated ratio of monoglutamates in folate accumulating crops [33,47], were incentives for engineering of the glutamate tail length. The polyglutamylation state could be manipulated towards accumulation of polyglutamates by knock-down of γ-glutamyl hydrolase (GGH), which removes the glutamate tail in folate homeostasis in vacuoles. This concept has been addressed in *Arabidopsis* and tomato, confirming that vacuolar *GGH* expression has a negative influence on folate content and polyglutamate levels [11]. Consequently, the level of polyglutamated folates is enriched upon GGH suppression, due to an enlarged vacuolar sink [11]. Conversely, overexpression of *FPGS*, responsible for the addition of the polyglutamate tail, resulted in a 4.7-fold increase in total folate of rice endosperm (Abilgos Ramos R, PhD thesis, University of Nottingham, 2010). Furthermore, ectopic expression of mitochondrial *FPGS* was proven successful in the endosperm of GA-engineered rice, resulting in an increase of polyglutamylated folates, exhibiting enhanced stability upon storage [35••]. This engineering strategy is, however, metabolically different, as it implies trapping an enlarged pool of folate polyglutamates in mitochondria, where they are more likely to be exposed to reactive oxygen species (ROS).

Folate binding proteins

Folate binding proteins (FBPs) have been of major interest for metabolic engineering strategies [38], as they are known to greatly stabilize folates in mammals [49]. Because plant derived FBPs remain to be characterized, current biofortification approaches rely on their

mammalian counterparts. In this perspective, synthetic codon-optimized bovine FBP was introduced in GA-engineered rice endosperm, enabling sequestration of folate polyglutamates in the cytosol [35••]. Rice lines obtained by this intervention possess improved folate stability, as well as folate levels exceeding those in the 'first generation' of folate biofortified rice [33]. Interestingly, engineering the folate binding glycine *N*-methyltransferase (GNMT) from rat liver was shown to be the most successful one-gene approach in rice endosperm, exhibiting 8.8-fold folate enhancement (Abilgos Ramos R, PhD thesis, University of Nottingham, 2010). Taken together, mammalian FBPs are suggested to augment folate levels by promoting their sequestration (creating a folate sink), additional to their ability to prolong crop storage by increasing folate stability.

Engineering folate homeostasis

Driving folate homeostasis towards accumulation of more stable folate forms has been proposed as an alternative engineering strategy [50]. 5-formylTHF is the most stable naturally occurring folate, with a presumed storage function [1,4]. Mutation of formylTHF cycloligase (5-FCL), the sole enzyme known to consume 5-formylTHF, led to enrichment of *Arabidopsis* leaves with folate, unfortunately coinciding with reduced growth rate [51].

Future research challenges

The occurrence of folate deficiency is predominantly caused by low folate levels in popular staples such as rice, potato, maize, plantain, cassava and wheat (Table 1). Biofortification via metabolic engineering or breeding holds the potential to reach the required folate levels in these crops, the concept of which has been proven in rice [35••].

Breeding

Future breeding strategies should focus on the pursuit of sufficient folate variation in target crop germplasm, followed by identification of the underlying QTLs. Despite the limitation of breeding strategies, high-resolution QTL-mapping in model species will enable identification of novel engineering targets for folate biofortification [25,32•,52] (Figure 3).

Metabolic engineering

Folate biosynthesis

Simultaneous activation of the *p*-ABA and pterin branches of folate biosynthesis appears essential to reach substantial folate enhancement, considering that single-gene approaches have only resulted in modest folate increase, due to inadequate supply of pterins and/or *p*-ABA. Therefore, a GA-engineering push-strategy will remain a prerequisite in folate biofortification [43•,53•]. Wheat is a good candidate crop for assessing this engineering approach, as it has been shown to harbor the complete active folate biosynthesis pathway in seeds [54]. This strategy, however, is not a guarantee for success, as its implementation remained ineffective in *Arabidopsis* and potato [45]. Hence, future biofortification strategies should include a back-up strategy, applicable to these crops in which GA-engineering approaches are ineffective (Figure 3). The latter should tackle the remaining hurdles impeding folate biofortification downstream of *p*-ABA and pterin accumulation, the existence of which has been observed in *Arabidopsis* and potato [45]. Co-occurrence of pterins and *p*-ABA has also been detected in G-engineered Mexican common bean [42••], suggesting intracellular transport of these intermediates or HPPK/DHPS activity to be possible constraints. The extent to which intracellular transport determines folate accumulation could be examined by assessing subcellular localization of folates, together with its biosynthetic intermediates, in the engineered plants. In this regard, characterization of a mitochondrial pterin importer could mean a leap forward towards optimized metabolic engineering of folate content. To date, only plastidial and vacuolar folate monoglutamate transporters have been identified [55–57]. On the other hand, the role of *HPPK/DHPS* and other biosynthesis genes, could be examined in transgene-stacking approaches, in combination with GA-engineering.

Folate stability

Concerning folate stability, different strategies need further assessment: (1) introduction of FBPs, (2) engineering glutamate tail length, (3) enhancing antioxidant levels, (4) augmenting folate salvage (Figure 3).

Considering the power of folate sequestration and protection by transgenic FBPs, targeting to different subcellular compartments could be tested, thereby fine-tuning the engineering strategy. Furthermore, different non-plant FBPs could show to be more successful than thus far implemented proteins, as their efficacy appears variable (Abilgos Ramos R, PhD thesis, University of Nottingham, 2010).

Extending the glutamate tail by heterologous expression or overexpression of cytosolic *FPGS* is of particular interest [35••], as it ensures folate accumulation in the cytoplasm (even stronger in combination with FBP), guarded from detrimental reactive oxygen species in the mitochondria or GGH activity in the vacuoles. Similarly, lowering GGH activity could be beneficial, given its ability to counteract the accumulation of polyglutamates in the cytosol [11,35••]. Novel techniques in genome editing, for example, the CRISPR/Cas9 technology [58], enable such directed manipulations of gene activity. However, GGH suppression should be approached with caution, given its role in folate salvage, forming *p*-ABA-Glu from *p*-ABA-Glu_n released upon (photo)-oxidative cleavage of THF-Glu_n. Therefore, in future metabolic

engineering, GGH could be altered to favor *p*-ABA-Glu_n as a substrate over THF-Glu_n, using directed evolution [59,60].

In biofortification, the most desired folate vitamer is the methylated derivative of the fully reduced THF (5-methyl-THF), given its stability and bio-activity. In this respect, enhancing antioxidant levels could assist in accumulation of the targeted folate vitamer together with a possible protection from oxidative cleavage [3]. This strategy could be reinforced by the capacity of folates to influence the cellular redox state, thereby ensuring sufficient replenishment of reduced antioxidants [61••]. Antioxidant candidates are ascorbate (vitamin C), pyridoxine (vitamin B6) and α-tocopherol (vitamin E), which could further enhance the nutritional value of the engineered crop [62,63].

Similarly, folate salvage, more particularly HMDHP recovery from DHPA by pterin aldehyde reductase (PTAR), has emerged as another possible target for folate biofortification. This is supported by the occurrence of pterin aldehydes in the G-engineered Mexican common beans [42••]. Interestingly, folate salvage could be extended, by introduction of a protozoan pterin reductase gene, capable of reducing fully oxidized pterins [64,65].

Conclusion

Folate biofortification exemplifies how metabolic engineering strategies enable the acquisition of fundamental knowledge on the complex matter of folate biosynthesis, salvage and homeostasis, as well as its regulation, part of which remains to be elucidated. The main goal is to design an effective biofortification strategy, considering both folate accumulation and stability, adaptable to the specific metabolism of different target tissues in crops cultivated in regions troubled with folate malnutrition. A strategy successful for biofortification of potato, combined with further fundamental research in *Arabidopsis*, could provide keys to effective folate enhancement in other staples. This could form a cornerstone for multi-biofortified crops [66], as these promise to be a powerful tool to reduce the global burden of micronutrient deficiency.

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