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DARWIN REVIEW

Present and future of folate biofortification of crop plants

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Abstract

Improving nutritional health is one of the major socio-economic challenges of the 21st century, especially with the continuously growing and ageing world population. Folate deficiency is an important and underestimated problem of micronutrient malnutrition affecting billions of people worldwide. More and more countries are adapting policies to fight folate deficiency, mostly by fortifying foods with folic acid. However, there is growing concern about this practice, calling for alternative or complementary strategies. In addition, fortification programmes are often inaccessible to remote and poor populations where folate deficiency is most prevalent. Enhancing folate content in staple crops by metabolic engineering is a promising, cost-effective strategy to eradicate folate malnutrition worldwide. Over the last decade, major progress has been made in this field. Nevertheless, engineering strategies have thus far been implemented on a handful of plant species only and need to be transferred to highly consumed staple crops to maximally reach target populations. Moreover, successful engineering strategies appear to be species-dependent, hence the need to adapt them in order to biofortify different staple crops with folate.

Key words: Biofortification, crops, deficiency, folate, folic acid, fortification, metabolic engineering, neural tube defects.

Introduction

Folates are a group of water-soluble B vitamins (B9), derived from tetrahydrofolate (THF), the most reduced folate form. THF contains three building blocks—the pteridine, *p*-aminobenzoate (*p*-ABA), and glutamate moieties—which are produced separately and subsequently joined (Fig. 1). Folates can differ in the length of the glutamate tail (ranging from one to approximately eight γ-linked L-glutamates and the one-carbon (C1) attached to the molecule (a methyl-, formyl-, methylene-, methenyl-, or formimino- unit) (inset Fig. 1). Each of them has a specific role in C1 metabolism (for a review, see Ravanel *et al.*, 2011). THF, and derivatives thereof, can only be synthesized *de novo* by plants and microorganisms. Thus, humans are entirely dependent on their diet to obtain the necessary amount of folates needed for a broad range of physiological and molecular processes. The recommended daily allowance (RDA) of folates is 400 μg for adults and 600 μg for pregnant women (National Institutes of Health, Office of Dietary Supplements: http://ods.od.nih.gov/factsheets/Folate-HealthProfessional/). Folates act as C1 donors and acceptors. In addition, folates play a central role in the biosynthesis and metabolism of nucleotides, amino acids (serine, glycine, histidine, and methionine) and pantothenate (vitamin B5) (Blancquaert *et al.*, 2010) and 5-methylTHF provides methyl units to methyltransferases, which use a broad range of substrates, such as hormones, DNA, proteins, and lipids, as part of the methyl cycle (Scott *et al.*, 2000). Green leafy vegetables, beans, and certain fruits are rich sources of folates, as are fermented products. However, most staple crops, although rich in starch content, contain a low folate level while populations consuming monotonous

Abbreviations: ADCS, aminodeoxychorismate synthase; C1, one-carbon; DALY, Disability-Adjusted Life Year; FBP, folate binding protein; FPGS, folylpolyglutamate synthetase; FW, fresh weight; GM, genetically modified; GTPCHI, GTP cyclohydrolase I; HMDHP, 6-hydroxymethyldihydropterin; HPPK/DHPS, dihydropterin pyrophosphokinase/dihydropteroate synthase; MTHFR, 5,10-methylenetetrahydrofolate reductase; NTD, neural tube defect; *p*-ABA, *para*-aminobenzoate; RDA, Recommended Daily Allowance; THF, tetrahydrofolate; WT, wild type.

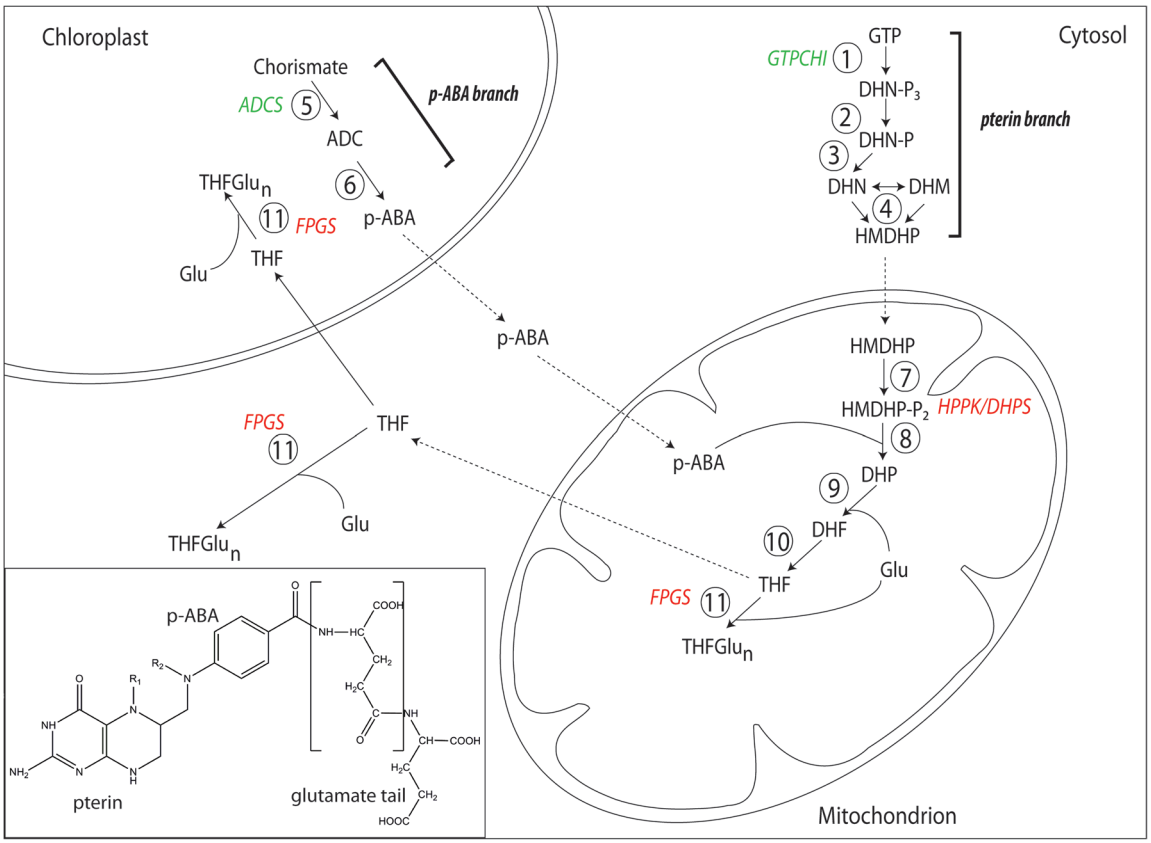

Fig. 1. The folate biosynthesis pathway in plants, characterized by its compartmentalization in the plastids, the cytosol, and the mitochondria and folate structure (inset). Dashed lines indicate hypothetical transport steps. Steps engineered for folate enhancement are indicated in green. Enzymatic steps suggested to be engineered are indicated in red. Inset: Folates are tripartite molecules, which consist of a pterin moiety, a *p*-ABA unit, and a glutamate tail. C1 units can be attached to R1 and/or R2. Compound abbreviations: ADC: aminodeoxychorismate; DHF, dihydrofolate; DHM, dihydromonapterin; DHN, dihydroneopterin; DHP, dihydropteroate; Glu, glutamate; HMDHP, hydroxymethyldihydropterin; THF, tetrahydrofolate. Enzymes: 1, GTP cyclohydrolase I; 2, dihydroneopterin triphosphate pyrophosphatase; 3, non-specific phosphatase; 4, dihydroneopterin aldolase; 5, aminodeoxychorismate synthase; 6, aminodeoxychorismate lyase; 7, hydroxymethyldihydropterin pyrophosphokinase; 8, dihydropteroate synthase; 9, dihydrofolate synthetase; 10, dihydrofolate reductase; 11, folylpolyglutamate synthetase.

diets, mainly consisting of these staple crops, often suffer from a suboptimal folate intake. Folate deficiency can cause neural tube defects (NTDs, such as spina bifida and anencephaly) (Geisel, 2003) and megaloblastic anaemia (Li *et al.*, 2003) and are associated with a higher risk on major depressive disorder (Papakostas *et al.*, 2012), Alzheimer disease (Seshadri *et al.*, 2002), cardiovascular (Scott and Weir, 1996) and coronary diseases (Stanger, 2004), stroke (Endres *et al.*, 2005), and several cancers (Choi and Friso, 2005). Several strategies are currently available to reduce folate deficiency, but they require educational efforts, changes in dietary habits and/or specialized infrastructure, making them difficult to implement in poorer regions of the world. Therefore, the biofortification of crops by metabolic engineering offers a good alternative in the battle against folate deficiency. In this paper, we focus on the global occurrence of folate deficiency and investigate which staple crops could be targeted to ameliorate folate intake in areas which suffer the most from folate deficiency. Next, metabolic engineering strategies that can be implemented to enhance folate levels in these staple crops are discussed. Furthermore, light is shed on the potential market demand and cost-effectiveness of folate-biofortified crops, taking rice as an example. Finally, the most important challenges in the biofortification of crops and its implementation that need to be addressed in the future are highlighted.

What is folate deficiency?

Before defining folate deficiency, it is important to consider some crucial processes of folate metabolism in the human body. Upon consumption, folates are released from the food matrix and taken up by the jejunal brush border. Here, polyglutamylated folates are first converted to their monoglutamylated forms by a deconjugase (also known as folylpolyglutamate carboxypeptidase) and subsequently transported into the inner layers of the mucosa. This transport occurs through a saturable process, but at local higher folate concentrations (>10 μmol l^{-1}), simple diffusion may occur as well (Gregory, 2001). In the mucosal enterocytes, these folates are converted to 5-methyltetrahydrofolate, which is released into the circulation system and transported throughout the human body. Folates are present in most body fluids and tissues, but the highest concentrations are found in the liver where folates are stored and redistributed to other tissues. In this way, stored folates in well-nourished adults may still provide the body for 4–5 months after a reduction of dietary

folate intake (Hercberg and Galan, 1992). Blood plasma transports the necessary amount of folates to all cells. During red blood cell maturation, the folate concentration found in erythrocytes reflects the blood serum folate level. When the latter is low, folate-deficient red blood cells replace the older, healthy erythrocytes which may lead to megaloblastic anaemia. Indeed, when dietary folate intake is reduced, folate concentrations in blood serum and erythrocytes start dropping within 3 weeks and 17 weeks, respectively, while the early symptoms of megaloblastic anaemia can be seen after 19 weeks (Herbert, 1962).

Since it is difficult to estimate dietary folate intake due to the complexities in monitoring dietary exposures and the concomitant measurement errors characteristic for these assays (Park *et al.*, 2013), folate deficiency is defined by folate concentrations in erythrocytes (on average <140 ng ml^{-1}) and/or blood plasma (<3 ng ml^{-1}) (Blount *et al.*, 1997) although, depending on the study, other thresholds are used as well. However, since a number of diseases and disorders related to folate deficiency are caused by elevated homocysteine levels, it is necessary to take concentrations of the latter into account as well. Methionine synthases convert homocysteine to methionine with 5-methyltetrahydrofolate and vitamin B12 as cofactors. Thus, in folate- and vitamin B12-deficient people, homocysteine can accumulate due to a low methionine synthase activity (Fig. 2). In a study on folate status of adolescent Nigerian girls, it was shown that subjects with normal blood folate levels still can have elevated homocysteine concentrations, most likely due to a low vitamin B12 status (VanderJagt *et al.*, 2000). In this respect, genetic factors may also cause elevated homocysteine levels (and thus lead to disorders caused by folate deficiency). A mutation in the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR), which converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, may result in a thermolabile MTHFR variant, with a reduced enzymatic activity which can result in an insufficient 5-methyltetrahydrofolate availability for methionine synthases and hence elevated homocysteine levels (Frosst *et al.*, 1995).

The global status of folate deficiency

Reports on folate deficiency are mainly focused on fertile women, and adults in general, as beneficiaries of folate strategies. The latter is well-documented in European (13 countries with at least one survey) and South-East Asian regional populations (six countries with at least one survey), while data on folate status at a national level and in other parts of the world are often incomplete or lacking (McLean *et al.*, 2008). At the national level, only nine countries are covered worldwide (McLean *et al.*, 2008). On the other hand, the occurrence of neural tube defects (NTDs), one of the most severe disorders caused by folate deficiency, is well known and, therefore, often used to map folate deficiency worldwide. Based on the total number of NTDs, as reported in UNICEF's Global Damage

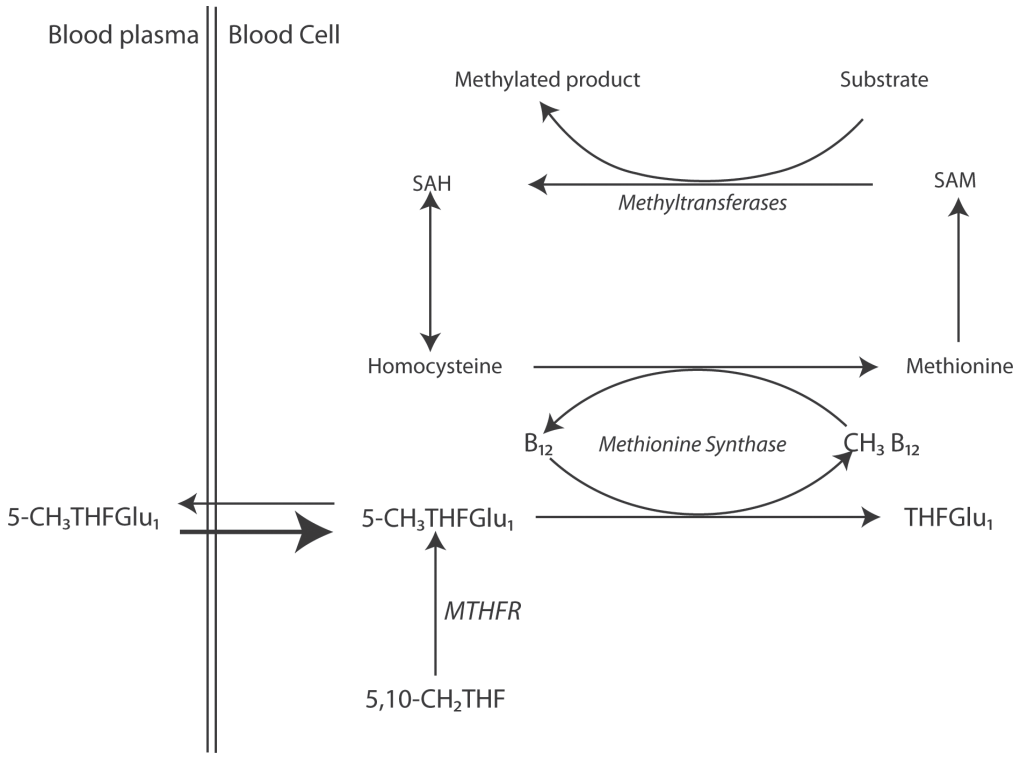

Fig. 2. Simplified representation of the methylation cycle in blood serum and cells. Enzymes are indicated in italics. Abbreviations: MTHFR: 5,10-methylenetetrahydrofolate reductase; B12, vitamin B12 (cobalamin); CH_3-B12, methylcobalamin; 5-CH_3THFGlu$_1$, 5-methyltetrahydrofolate monoglutamate; 5,10-CH_2THF, 5,10-methylenetetrahydrofolate; THFGlu$_1$, tetrahydrofolate monoglutamate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

Assessment Report of Vitamin and Mineral Deficiency (UNICEF, 2004), and the World Bank demographic databases on total population and birth rate (World Bank, 2011), the prevalence of NTDs is calculated. Figure 3 provides an overview of the key areas of high NTD prevalence, defined as >10 NTDs per 10 000 births, reflecting folate deficiency. Out of the 86 selected countries, 24 have a prevalence rate above 20 per 10 000 births. Within the top 20 countries, the population size of seven countries is below 1 million. Amongst these, Bhutan is the only country with an NTD prevalence rate above 30 (i.e. 101.3). When excluding these smaller countries, the five highest ranked countries are all located in South-East Asia: Cambodia, Bangladesh, Myanmar, Thailand, and China. Given that China and Bangladesh are among the world's most populated areas, there is still a large burden of folate deficiency in these countries. According to the 'March of the Dimes' global report on birth defects (Christianson *et al.*, 2006), an annual number of 300 000 NTDs occur worldwide.

In countries with a high NTD prevalence, dietary folate intake is assumed to be suboptimal. In poorer regions of the world, this is often caused by the consumption of a monotonous diet which mainly consists of folate-poor staple foods, such as cereals, potato, cassava, and banana. Therefore, to understand fully why NTD occurrence rates are high in those poorer regions and to design strategies to fight folate deficiency, it is essential to map staple crop growth areas and consumption globally. The most important staple crops (ranked by production) are listed in Table 1. In the five aforementioned countries with the highest NTD occurrence, rice, wheat, potato, cassava, and plantain are the most consumed staple crops, with rice being most produced and consumed (Table 2). Although these staple crops are rich sources of carbohydrates, they contain limited amounts of folate (Table 3). Ergo, it is not surprising that folate deficiency is much more pronounced in developing regions of the world.

The world's most produced staple crop is maize (*Zea mays* L.), with a global production of 856 million tons in 2012 (USDA Foreign Agricultural Service: http://www.fas.usda.gov/), of which 274 and 205 million tons were harvested in the United States of America and China, respectively. However, despite its high ranking in staple food production, only 3% of US-grown maize is used for human consumption (AgMRC: http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/white-corn/). In China, about 10% is grown for human consumption (Huang and Rozelle, 2006). Nutritionally, maize contains high levels of carbohydrates [74.26 g per 100 g fresh weight (FW)], but folate content is low (19 µg per 100 g FW) (USDA-ARS, 2012).

Rice (*Oryza sativa* L.) is the second most produced cereal crop, which provides up to 80% of the daily caloric intake to approximately 3 billion people. In 2011, 723 million tons of rice were produced, of which 90% in Asia (FAO, 2011). Since most of the nutrients and vitamins are concentrated in the outer layers of brown rice, milling (removal of the fat-rich husk and bran layers to obtain white rice) greatly reduces its nutritional value. White rice is a rich source of carbohydrates (79.34 g per 100 g FW), but extremely poor in vitamins containing only 9 µg of folate per 100 g FW (USDA-ARS, 2012). Therefore, rice-consuming populations in developing regions often suffer from persistent folate deficiency (Cherian *et al.*, 2005; Li *et al.*, 2006).

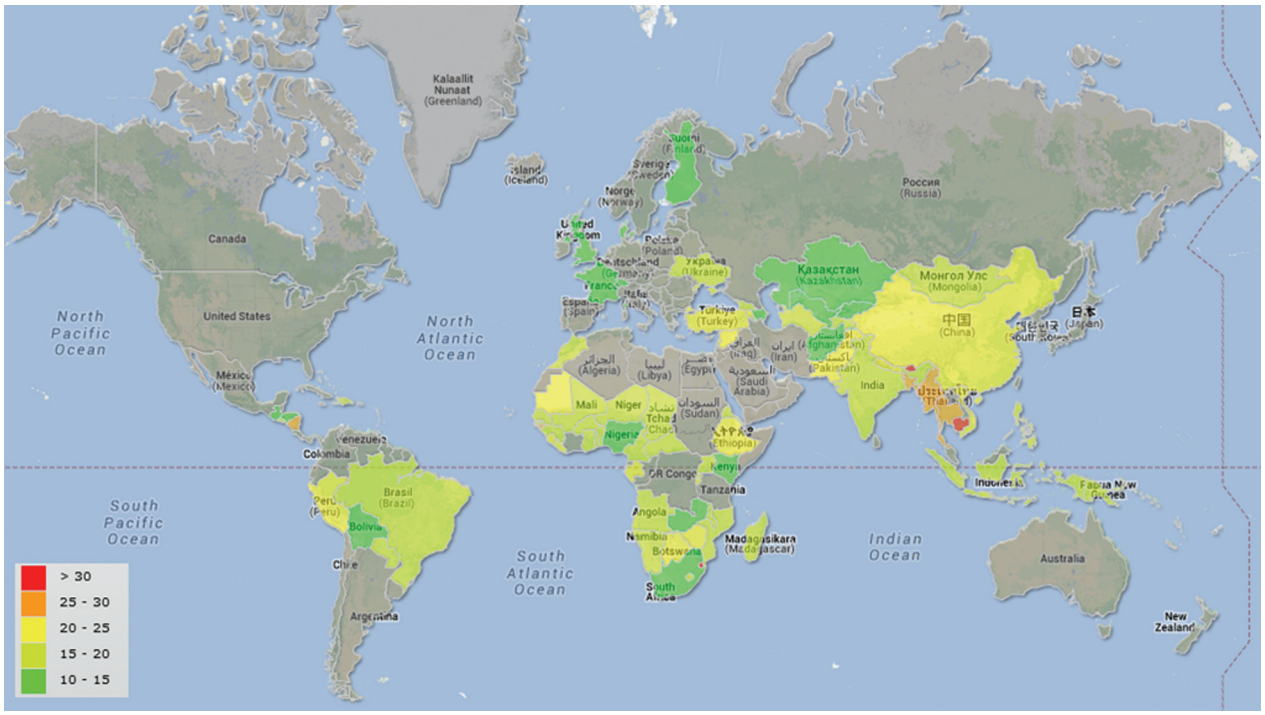

Fig. 3. Risk regions of folate deficiency based on NTD prevalence (expressed as number of NTDs per 10 000 births). Own compilation by TargetMap, based on: World Bank (2011), UNICEF (2004), and EUROCAT (2011).

Table 1. *The most important staple crops worldwide, ranked by production*

Data are derived from FAO (2011).

Staple crop	Production(million tons)	Growth area(million ha)	Main producing countries
Maize	883	170	USA (35%), China (22%)
Rice	723	164	China (28%), India (22%)
Wheat	704	220	China (17%), India (12%)
Potatoes	374	19	China (24%), India (11%)
Soybeans	261	103	USA (32%), Brazil (29%), Argentina (19%)
Cassava	252	20	Nigeria (21%), Brazil (10%), Indonesia (10%)
Barley	134	49	Russia (13%), Ukraine (7%)
Bananas	107	5	India (28%), China (10%)
Sweet potatoes	104	8	China (72%)
Yams	57	5	Nigeria (65%)

Table 2. *Top five countries with the highest NTD prevalence (Buthan excluded)*, with the most important staple crops produced and consumed (in 2011)*

Population data are derived from the World Bank (2011), NTD prevalence from UNICEF (2004), data on staple crops from FAO (2011).

Country	Population (million)	NTD prevalence (per 10 000 births)	Most important staple crops produced (million tons)	Consumed per capita (kg yr^{-1})
China	1344.1	23.70	Rice (202.7), maize (192.9), wheat (117.4)	Rice (76.3), wheat (66.4)
Thailand	69.5	26.69	Rice (34.6), cassava (21.9)	Rice (133), wheat (16.8)
Myanmar	48.3	27.94	Rice (32.8)	Rice (140.8), plantain (13.7)
Bangladesh	150.5	27.96	Rice (50.6), potatoes (8.3)	Rice (173.3), potatoes (29.4)
Cambodia	14.3	30.01	Rice (8.8), cassava (4.3)	Rice (160.3), cassava (25.2)

*Buthan has the highest NTD prevalence rate worldwide (101.3 per 10 000 births), but was excluded from this analysis due to its low birth rate and population number.

Table 3. *(a) Folate, cobalamin (vitamin B12), and iron composition per 100 g of various staple foods; (b) a few examples of folate-rich vegetables (μg per 100 g FW) and their iron content (mg per 100 g FW)*

All values were obtained from the USDA National Nutrient Database for Standard Reference, Release 25 (USDA-ARS, 2012).

(a)

Food	Folate (μg)	Vitamin B12 (μg)	Fe (mg)
Corn, sweet, white, raw	46	0	0.52
Corn, yellow	19	0	2.71
Rice, white, unenriched	9	0	0.8
Wheat flour, unenriched	26	0	1.17
Bread, wheat	85	0	3.46
Potato, raw	16	0	0.78
Cassava, raw root	27	0	0.27
Plantains, raw	22	0	0.6

(b)

Food	Folate (μg)	Fe (mg)
Mung beans, mature, raw	625	6.74
Lentils, cooked	180	3.33
Chickpeas, cooked	172	2.89
Soybean, green, raw	165	3.55
Spinach, cooked	146	3.57
Broccoli, cooked	108	0.67

Wheat (*Triticum* ssp.) is the third most produced staple crop worldwide. It is a daily source of calories, proteins (Seilmeier *et al.*, 1991) and micronutrients (especially in wholegrain products) for the majority of the world population (Shewry, 2009) and ensures 70% of the caloric intake of rural populations. Approximately 30% of global wheat production is located in China and India (FAO, 2011). Folate concentration in unenriched wheat flour and wheat bread is 26 and 85 μg per 100 g FW, respectively, making wheat a poor folate source (USDA-ARS, 2012).

The cultivated potato (*Solanum tuberosum* L.) originated in South America, where it has been grown as a staple crop for over 10 000 years. Approximately 46% of potato production in 2011 was located in Asia, while 35% was produced in Europe (FAO, 2011). Potato has a high carbohydrate (17.47 g per 100 g FW) and vitamin C content (19.7 mg per 100 g FW), but is a poor source of folates (19 μg per 100 g FW) (USDA-ARS, 2012).

Cassava (*Manihot esculenta* Crantz) is a vital source of calories, representing one-third of the daily caloric intake for approximately 500 million people in over 105 countries. The main growth areas are located in sub-Sahara Africa, Asia, and South-East Asia (El-Sharkawy, 2003). Global cassava production in 2011 was approximately 252 million tons (FAO, 2011). In Africa, cassava is mainly cultivated for food (141

million tons in 2011 (FAO, 2011)), while it is used in animal feed and for industrial purposes in Asia and South-East Asia. Although cassava roots are a rich source of carbohydrates (38.06 g per 100 g FW) and vitamin C (20.6 mg per 100 g FW) (Montagnac *et al.*, 2009), they contain only 27 µg of folate per 100 g FW (USDA-ARS, 2012).

The battle against folate deficiency

Several strategies can be developed to improve folate intake levels. The production and consumption of folate-rich food sources is the ideal way to prevent folate deficiency. Beans, for instance, are rich sources of folate, other vitamins, and nutrients. However, promoting the consumption of folate-rich food sources often implies educational efforts and changes in dietary habits. In poorer regions, diet diversification and education are not for granted and folate-rich products are often inaccessible or unaffordable for populations at risk, whose diet mainly consists of staple crops. Folic acid supplementation by pills can be very helpful in preventing NTDs. However, since the formation and closure of the neural tube takes place within the first 3–4 weeks after conception, before most women are aware of their pregnancy, this strategy has shown limited success. Half of the pregnancies are unplanned in the United States, a number which is slightly reduced to 44% in Europe (Singh *et al.*, 2010). Hence folic acid supplementation alone is inadequate to prevent NTDs. The use of folic acid pills in NTD prevention requires general basic knowledge on the action of this vitamin and thus public health campaigns to promote this strategy. In the United States, almost half of the target women took folic acid pills after a health campaign; however, most women were not aware about the need to take these pills in the periconceptional phase (Sillender and Pring, 2000). Unfortunately, although older women planning a pregnancy are receptive to folic acid recommendations, young women, who are not thinking about becoming pregnant in the near future, showed no interest in folic acid as a way to prevent NTDs (Rofail *et al.*, 2012). Therefore, it was advised to adapt these health campaigns to promote folic acid administration as part of a 'healthy lifestyle' rather than as a periconceptional supplement (Rofail *et al.*, 2012). While most countries introduced a folic acid supplementation policy, which is the common folate strategy in the EU (EUROCAT, 2009), folic acid food fortification, either mandatory (e.g. United States, Canada, and Australia) or voluntary (food product producers have the choice to use fortified ingredients, such as flour) (e.g. New Zealand and a few EU countries) (Czernichow *et al.*, 2005; McLean *et al.*, 2008), is increasingly used by many countries to fight folate deficiency, for example, 74 countries currently enrich flour with folic acid (Flour Fortification Initiative, 2012: http://www.ffinetwork.org). However, despite their success, it seems that folic acid programmes in risk regions are either lacking or less successful. While folic acid fortification has shown its effectiveness in developed, high-income countries, such as Canada and the United States (Choumenkovitch *et al.*, 2002; De Wals *et al.*, 2007), it still needs to be determined whether the implementation of this and other folic acid programmes in poor, rural areas with low socio-economic status is the more cost-effective, sustainable, and practically feasible option to reduce folate deficiency and its negative health outcomes, such as NTDs. Obviously, folic acid supplementation and food fortification require a specialized infrastructure, which makes it difficult to implement in poorer regions worldwide.

Adverse effects of folic acid supplementation

Scientific concern is growing about folic acid supplementation and fortification, because high folic acid intake could have adverse effects on human health, as for instance an increased risk of prostate and colorectal cancer (Cole *et al.*, 2007). Moreover, high doses of folic acid may compromise the effectiveness of anti-folate drugs used in treatment against cancer, rheumatoid arthritis, and psoriasis (Arabelovic *et al.*, 2007). Since folic acid needs to be reduced and methylated to its biologically active form 5-methyltetrahydrofolate, intake of a single high dose of folic acid (>300 µg) can result in the circulation of unconverted folic acid in the human body. Although the effects thereof are still poorly known, it may promote tumour growth and mask pernicious anaemia, especially in combination with cobalamin (vitamin B12) deficiency (concentration of serum B12 <148 pmol l^{-1} or <200 pg ml^{-1}) (Scott *et al.*, 2000). Cognitive impairment and anaemia were associated with high serum folate concentrations in combination with vitamin B12 deficiency in the elderly (Morris *et al.*, 2007). High folic acid intake may even mask the diagnosis of B12 deficiency (Mills *et al.*, 2003). Moreover, folic acid supplementation to cobalamin-deficient women may also impair fetal growth and brain development (Takimoto *et al.*, 2011; Marean *et al.*, 2011) and could be harmful to the nervous system when combined with vitamin B12 deficiency (Reynolds, 2006). The negative effects of folic acid (over)consumption are unlikely to occur with natural folates, since the latter act differently on folate metabolism (Ross *et al.*, 1984). Indeed, a higher risk of breast cancer was reported upon intake of high doses of folic acid but not with natural folates (Stolzenberg-Solomon *et al.*, 2006) and a similar report was published on colorectal cancer (Sanjoaquin *et al.*, 2005). More and more researchers promote the intake of natural folates instead of folic acid (Ulrich and Potter, 2006) and fermentation of food by, for example, lactic acid bacteria has been suggested as a way to enhance natural folate levels in food (Iyer and Tomar, 2009). The question remains whether a folic acid fortification policy, which could be beneficial for a small group of people (in the prevention of NTDs), is justifiable to expose a whole population, considering the growing evidence of the adverse effects of an increased folic acid intake (Smith *et al.*, 2008).

Both vitamin B12 and folates are necessary in the conversion of homocysteine to methionine (catalysed by methionine synthase) (Fig. 2); therefore, improving folate status, in theory, will not necessarily decrease serum homocysteine levels in vitamin B12-deficient humans and, vice versa, cobalamin deficiency will lead to an accumulation of

5-methyltetrahydrofolate (termed the methyl-folate trap), leading to an increase in serum folate levels. Conversely, in a folate-deficient background, methylation is inhibited due to the absence of methyl groups, resulting from a low methionine pool. The assumption that both vitamin levels need to be normal to lower the homocysteine level was confirmed in younger people (<60 yrs), but not in older subjects (>60 yrs), where homocysteine concentrations indeed dropped with high serum folate levels (Solomon, 2013). Molluscs, fish, meat, and dairy products are rich cobalamin sources (USDA, 2012), while staple crops are almost devoid of vitamin B12 (Table 3). Hence, for populations mainly consuming staple crops it is difficult to meet the RDA of vitamin B12 (2.4 μg for an adult and 2.8 μg for pregnant women (Shibata *et al.*, 2013).Consequently, vitamin B12 deficiency is a global problem as well. Although folate and vitamin B12 are important in the prevention of anaemia, iron deficiency is the most common aetiological factor causing anaemia, which affects approximately 1.6 billion people worldwide, mostly in African and Asian regions (Bhutta *et al.*, 2010). The latter is not surprising, since staple foods contain only small traces of iron (Table 3), while male adults need 8 mg and fertile women 18 mg of iron per day. The RDA of iron for pregnant women is even 27 mg (Institute of Medicine of the National Academies, 2001). Importantly, folate and vitamin B12 deficiency may mask iron deficiency in patients with active rheumatoid arthritis, who often suffer from anaemia (Vreugdenhil *et al.*, 1990). This illustrates the need to fight folate deficiency as a silent killer affecting many more people than those estimated by NTD prevalence alone.

Current progress in folate biofortification of crops

In theory, the biofortification of staple crops with folate can be achieved by two approaches: through conventional breeding or through metabolic engineering. Plant breeding relies on the ability of two plants to cross with each other and produce fertile descendants. Those prerequisites can only be met between closely related species, which limits the extent of biofortification, due to its reliance on the natural variation of the trait (in this case folate levels) between the parental species. Indeed, studies on variation in folate content between different rice (Blancquaert *et al.*, 2010) and wheat (Piironen *et al.*, 2008) genotypes showed a 2-fold difference in variation. Hence, plant breeding alone appears insufficient to enrich those species and, most likely, other important staple crops as well, in the battle against folate deficiency. In addition, plant breeding can be very time-consuming, although it can be facilitated and accelerated by quantitative trait loci mapping, in combination with marker-assisted breeding. Metabolic engineering can be used as a strategy to enhance folate levels in crops with limited variation in folate content. High folate lines will have to be produced for locally adapted varieties of each crop, either by multiple transformation or by crossing high folate germplasm into agronomically useful varieties. Field trials would then be needed to assess the consequences for yield and disease resistance. In terms of the dissemination of fortified varieties, the CGIAR institutes (http://www.cgiar.org) will play an important role in the early breeding and distribution of germplasm to national breeding programmes in developing countries. However, although a successful proof-of-concept for folate biofortification is available in laboratory varieties and cultivars of certain target crops (e.g. for rice: Storozhenko *et al.*, 2007; and tomato: Diaz de le Garza *et al.*, 2007) or will become available in the future, the path from laboratory to market is long, expensive, and loaded with regulatory and political hurdles. More than a decade after the creation of Golden Rice (GR) in 2000 (Ye *et al.*, 2000), and further improvement in 2005 (Paine *et al.*, 2005), GR is still not available for consumption, although its release has already been approved several times. The regulatory steps that need to be followed upon approval are well-described (http://www.goldenrice.org/Content2-How/how4_regul.php) (see also König *et al.*, 2004; Magana-Gomez and de la Barca, 2009) and GR will probably act as the pioneer to open the gate for the approval of other engineered crops for human consumption. Unfortunately, GR, and the second generation of Genetically Modified Organisms (GMOs), in general, suffer from a rather negative public opinion, mainly caused by the first generation of GMOs. Should this first generation have been focused on improved nutritional traits instead of lowering farm-level production costs, GR and other nutritionally improved crops may already have been publicly accepted and available for their target consumers. Once the road is cleared by GR rice, folate-biofortified crops could become available to target populations within five years.

The folate biofortification of crops through metabolic engineering can offer a sustainable alternative to the aforementioned strategies to fight folate deficiency, especially for poor populations in rural remote areas. Thus far, metabolic engineering was applied solely through the over-expression of key folate biosynthesis genes (Fig. 1). Over the past decade, engineering attempts were reported in *Arabidopsis* (Hossain *et al.*, 2004; Blancquaert *et al.*, 2013a), tomato (Diaz de la Garza *et al.*, 2004, 2007), rice (Storozhenko *et al.*, 2007), lettuce (Nunes *et al.*, 2009), white corn (Naqvi *et al.*, 2009), and potato (Blancquaert *et al.*, 2013a) (see Supplementary Table S1 available at *JXB* online). These attempts can be divided into two groups: (i) the over-expression of GTP cyclohydrolase I (GTPCHI) (resulting in G lines), the first enzyme in the pterin branch of folate biosynthesis, and (ii) the combined over-expression of GTPCHI and aminodeoxychorismate synthase (ADCS), the first enzyme in the *p*-ABA branch (resulting in GA or G+A lines, depending on whether both genes were combined on the same T-DNA or separately transformed lines were crossed). Plants over-expressing GTPCHI alone indeed had a massive increase in pterin levels (up to 1250-fold in *Arabidopsis* (Hossain *et al.*, 2004), which coincided with a 2–8.5-fold increase in folate content (the highest increase being reported in lettuce (Nunes *et al.*, 2009)). In these lines, a further folate enhancement was hampered due to a depletion of the *p*-ABA pool. Therefore, co-expression of both GTPCHI and ADCS was successful in tomato fruit (Diaz de la Garza *et al.*, 2007) and rice

seeds (Storozhenko *et al.*, 2007), where folate contents of up to 25-fold (tomato) and 100-fold (rice) the WT levels could be detected. Interestingly, *p*-ABA and pterin levels in these transgenics were still elevated compared with their respective wild types, indicating that an additional bottleneck is present against the higher accumulation of tetrahydrofolate. Attempts to biofortify *Arabidopsis* plants and potato tubers by enhancing both pterin and *p*-ABA levels did not result in high folate enhancement (Blancquaert *et al.*, 2013a). These data suggest that the two-gene strategy cannot be universally applied to biofortify crops with folate and that engineering strategies should be adapted in order to reach this goal (see paragraph 'Biofortifying staple crops with folate' in the section 'Future challenges').

Socio-economic potential of folate biofortified crops

Like any other biofortified crop developed through metabolic engineering, none of the folate biofortified crops has been approved for commercial release. Apart from the potential regulatory and post-approval hurdles associated with such GM food crops with health benefits (Potrykus, 2010), introducing folate biofortification will only be successful if there is sufficient demand, and if the burden of folate deficiency would be effectively reduced. In other words, in order to estimate the value of the promising efforts of folate biofortification and its potential as an alternative folate strategy, it is important to examine whether target populations would be in favour of GM crops with an enhanced folate content, or even willing to pay more for it, and whether the benefits of its introduction outweigh the costs. Previous socio-economic studies in this field focused on folate-biofortified rice in China. According to a 2010 population-based consumer study in Shanxi Province, China's most important risk region of neural-tube defects, this crop is generally well-accepted with about 62% willing to consume it compared with a relative small group of reluctant consumers (11%) (De Steur *et al.*, 2010a). When looking at women of child-bearing age as key beneficiaries of folate interventions, another study in Shanxi found that they are prepared to pay about 33% and 16% more for folate-enriched rice than for, respectively, regular rice or rice sold together with folic acid supplements (De Steur *et al.*, 2013a). But, more importantly, awareness of its biotech nature did not negatively affect consumers' preferences (De Steur *et al.*, 2013b).

By applying the Disability-Adjusted Life Year (DALY) framework, health impact studies (De Steur *et al.*, 2010b) as well as cost-effectiveness analyses (De Steur *et al.*, 2012) demonstrated the large potential value of implementing folate-biofortified rice in China. While the current burden of folate deficiency is significantly smaller than for other key micronutrient deficiencies (vitamin A, zinc, and iron), the potential reduction, through this agriculture-based intervention, is among the largest, i.e. 20–60%. Moreover, it would only cost between US$21 and US$64 to save a DALY with folate-biofortified rice, which further underlines its high cost-effectiveness (De Steur *et al.*, 2012). The low recurrent costs and the high folate levels after biofortification largely account for these promising figures. Furthermore, when incorporating folate in a multi-biofortified crop, the scope of the intervention is expanded to micronutrient malnutrition as a whole, which further increases the health benefits and improves its cost-effectiveness. However, its success might be dependent on the selected gene-stacking approach, for example, combining several micronutrient traits in one gene construct or combining separately developed and deregulated traits through crossing.

Future challenges

Biofortifying staple crops with folate

Since most staple crops are poor sources of folates and folic acid interventions are difficult to implement in poorer regions of the world, biofortification of these crops by metabolic engineering is currently the most promising approach to alleviate the global burden of folate deficiency. As mentioned above, a two-gene strategy gave the best results so far, but outcomes can be species-dependent. Although the new bottleneck in the flux toward folate enhancement has not been identified yet, an educated guess can be made. The biosynthesis of tetrahydrofolate in the mitochondria requires the import of both pterin and *p*-ABA in this cellular compartment (Fig. 1). Although folate enhancement could be compromised due to insufficient import of these precursors, this assumption is unlikely, since *p*-ABA could enter the mitochondria by simple diffusion (Quinlivan *et al.*, 2003) and the existence of plant pterin transporters has been suggested (Hanson and Gregory, 2011). Assuming that pterin and *p*-ABA import are not rate-limiting, the new constraint in tetrahydrofolate biosynthesis needs to be located downstream. It is well-known that the first enzymatic steps in biosynthetic pathways are often regulatory, allowing flux control toward target compounds. Indeed, the bifunctional enzyme dihydropterin pyrophosphokinase/dihydropteroate synthase (HPPK/DHPS), which catalyses the first mitochondrial reaction in THF biosynthesis (the activation of 6-hydroxymethyldihydropterin (HMDHP) to HMDHP pyrophosphate (HMDHP-P2) (HPPK activity) and the subsequent coupling with *p*-ABA to form dihydropteroate (DHPS activity)) is predicted to be an important regulatory enzyme, since its DHPS domain is inhibited by dihydropteroate (DHP), dihydrofolate monoglutamate ($DHFGlu_1$), and tetrahydrofolate monoglutamate ($THFGlu_1$), which are intermediates of the folate biosynthesis pathway (Mouillon *et al.*, 2002). Therefore, adapting engineering strategies to include over-expression of mitochondrial HPPK/DHPS, in combination with GTPCHI and ADCS, could be successful in improving the folate content in recalcitrant crops in which the two-gene strategy is inadequate, but also in other crops where a two-gene strategy works, in order to decrease the remaining high pterin and *p*-ABA levels and further enhance folate content. The latter could be important, since little is known about the possible toxicity of pterins (*p*-ABA is assumed to be harmless).

Folylpolyglutamate synthetase (FPGS), an enzyme which lengthens the glutamate tail of tetrahydrofolate, could also be a good candidate for engineering. Since this protein catalyses the last reaction in THF biosynthesis, its over-expression, in combination with GTPCHI and ADCS, could change engineering approaches from a 'pushing' to a 'pulling' strategy, hence forcing flux toward THF accumulation and possibly preventing pterin and *p*-ABA accumulation.

It is possible that a combined over-expression of GTPCHI, ADCS, and mitochondrial HPPK/DHPS is still insufficient to enhance folate levels in recalcitrant crops, due to an accumulation of DHP, $DHFGlu_1$, and/or $THFGlu_1$, inhibiting DHPS activity (Mouillon *et al.*, 2002). In this case, a quadruple gene strategy, in which the three aforementioned genes are over-expressed together with mitochondrial FPGS, could be successful. On the other hand, numerous examples exist where the removal of the regulatory domain was necessary to avoid feedback inhibition by intermediates and achieve accumulation of a target compound (e.g. AtTPS1 truncated at the N-terminal region to accumulate trehalose in yeast: Van Dijck *et al.*, 2002). However, since little is known about the regulatory and catalytic domains in DHPS, over-expression of a truncated mitochondrial HPPK/DHPS could have negative outcomes.

The question remains as to which engineering strategy should be applied to engineer folate levels in a staple crop successfully. Clearly, further research is required to provide a satisfactory answer, but it could be found by considering the following question: why was folate enhancement in tomato and rice successful with a two-gene approach while failing in potato and *Arabidopsis* (Blancquaert *et al.*, 2013a)? It is clear that the activity of endogenous downstream steps was insufficient to guarantee THF accumulation in these two species. Whether this is caused by species-specific regulatory mechanisms of the biosynthesis pathway or by an insufficient activity of downstream enzymes, remains to be proven. In tomato fruit it was shown that folate enhancement up-regulated the expression of the endogenous mitochondrial FPGS (Waller *et al.*, 2010), suggesting that unaltered FPGS activity would have compromised the outcomes. However, the expression of folate biosynthesis genes was not altered in biofortified rice (Blancquaert *et al.*, 2013b), implying that the basal expression of endogenous genes downstream of the engineered steps was sufficient to obtain high folate levels in rice seeds. A possible explanation can be found in species-specific and/or tissue-specific feedback mechanisms, compromising flux toward THF enhancement. Folate accumulation was targeted to endosperm (in rice) and pericarp (in tomato); tissues without the ability to differentiate into other tissues and whose primary function is to protect (endocarp) and supply the necessary nutrients and energy (pericarp and endosperm) to the embryos. On the other hand, potato tubers are derived from stem tissue (stolons) and besides being an important starch storage organ, it has the ability to redifferentiate and form new shoots; hence, it has a meristematic character. Since folate demand in meristematic plant tissue is high (due to its importance in C1 metabolism), it can be assumed that folate biosynthesis is tightly regulated in these cells, which could imply that engineering strategies should be adapted accordingly. A similar explanation for the unsuccessful attempts in *Arabidopsis* plants can be found in the importance of folates in photorespiration and chlorophyll biosynthesis, where C1 metabolism, and thus the demand of folate, should be tightly controlled in green tissues. Hence, in theory, in the biofortification of wheat, maize, barley (endosperm), and banana (mesocarp) a two-gene approach, only over-expressing GTPCHI and ADCS, should be sufficient to obtain high folate levels, whereas at least a three-gene strategy (GTPCHI and ADCS in combination with, for example, mitochondrial HPPK/DHPS and/or FPGS) would be required to enhance folate content in cassava (root) and potato.

Enhancing folate stability

Another important issue with respect to folate biofortification is folate stability, since obtaining high levels of this vitamin would be meaningless if they drop to basal levels upon food storage and processing. Indeed, folates are unstable compounds, susceptible to oxidative and photo-oxidative catabolism (Scott *et al.*, 2000) and degradation by pH variations (most folates are stable at pH 4–8 at 37 °C, except THF and dihydrofolate) (De Brouwer *et al.*, 2007)). Food processing, such as cooking reduced the levels of folate in biofortified rice by half (Storozhenko *et al.*, 2007). Therefore, these factors need to be taken into account when considering engineered staple crops as a way to fight folate deficiency. A few approaches have been suggested to improve folate stability (Blancquaert *et al.*, 2010): (i) engineering toward the accumulation of a more stable compound, (ii) simultaneous accumulation of compounds with a protective mode of action (e.g. anti-oxidants such as ascorbate), (iii) engineering compound salvage and breakdown reactions, and (iv) protein complexation. In folate biofortified rice, 5-methyl THF is the most abundant folate form (Storozhenko *et al.*, 2007). The most stable naturally occurring folate is 5-formyl THF. Engineering toward the accumulation of this folate derivative could enhance folate stability, but is undesirable, since no function in C1 metabolism has been attributed to this compound so far. Since polyglutamylation enhances the anionic nature of the vitamin, hence improving cellular retention, increasing the ratio between folate polyglutamates and monoglutamates could enhance its stability. Moreover, folate-dependent enzymes have a preference for binding with folate polyglutamylated forms and protein activity is positively correlated with the length of the glutamate tail (Shane, 1989). In WT rice seeds half of the folate pool is polyglutamylated, whereas only 10% is in engineered rice (Storozhenko *et al.*, 2007). Polyglutamylation could be enhanced by the over-expression of FPGS. Hence, a three gene strategy (GTPCHI, ADCS, and FPGS) could be beneficial to improve both folate accumulation and stability. Nevertheless, as aforementioned, since folate polyglutamates are converted to their monoglutamylated forms in the intestinal brush border prior to absorption, enhancing the poly/mono ratio of the folate pool in biofortified crops could compromise its bio-availability. Complexation of folates with folate binding proteins (FBP)

has been suggested (Storozhenko *et al.*, 2005). Unfortunately, although quite some research has been conducted on these proteins in mammals, their existence in plants remains to be proven. Nevertheless, mammalian FBP could be an excellent candidate to enhance folate stability in biofortified crops.

The creation of multivitamin/nutrient crops—stacking with other traits

Although numerous attempts on crop biofortification with micronutrients or vitamins have been reported (Fitzpatrick *et al.*, 2012), most of them describe the enhancement of a single compound. Nevertheless, multinutrient and multivitamin deficiencies represent a global issue compromising human health, hence the creation of engineered crops aimed at the enhancement of different vitamins and nutrients levels is desirable. To date, the only successful example of a multivitamin approach using metabolic engineering was the creation of biofortified white corn with enhanced levels of ascorbate, β-carotene and folate (Naqvi *et al.*, 2009). Since folate, cobalamin, and iron deficiency are important factors causing anaemia, the improvement of content of all three compounds in staple crops is a straightforward way to fight this disorder. In order to enrich the edible fraction of staple crops with iron, it is necessary fully to understand its metabolism in plants. This involves the uptake of iron by the roots, the transport to and accumulation in target tissues (Bhullar and Gruissem, 2013). Iron occurs in the soil as Fe^{3+} and Fe^{2+} and plants acquire Fe^{3+} from the soil by two strategies (for a review see Palmer and Guerinot, 2009). Several attempts to increase the iron content of rice seeds indicate that all the steps of iron uptake and transport into the seeds are required to successfully enhance iron levels in rice grains. A combinatorial approach, in which iron uptake and storage in rice seeds was engineered by the over-expression of *Arabidopsis* NAS (constitutive), common bean ferritin, and *Aspergillus fumigatus* phytase (both endosperm-specific expression) enhanced iron content of white rice seeds by 6-fold (Wirth *et al.*, 2009). These data not only suggest that different aspects of iron metabolism in plants need to be engineered in order to biofortify crops with iron, but also that tissue-specific expression of specific transporters and binding proteins, such as ferritin, is necessary to translocate/sequestrate iron into the target tissue.

Plants are not capable of biosynthesizing vitamin B12 *de novo*, only bacteria and *archaea* possess the necessary genes. Hence, improving cobalamin levels in crop plants is only possible by encouraging symbiosis between plants and bacteria and engineering the relocation of vitamin B12 into target tissues.

Conclusions

Folate malnutrition is an underestimated global problem and the currently implemented approaches to solve it are insufficient and can turn out to have adverse outcomes in the future. Folate biofortification of the most important staple crops by metabolic engineering could be a cost-effective, sustainable solution to eradicate folate deficiency worldwide. A better understanding of folate metabolism and, more specifically, its biosynthesis and regulation in different crops and tissues, is necessary to develop adequate engineering strategies to enhance folate levels in these plant species.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1: Overview of the current status of folate biofortification in plants. Abbreviations: ADCS, aminodeoxychorismate synthase; At, Arabidopsis thaliana; DW, dry weight; Ec, Escherichia coli; FW, fresh weight; GTPCHI, GTP cyclohydrolase 1.

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